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The applicant has subsequently filed a sequence listing and declared, that it includes no new matter.

(54) Genes encoding truncated cellulases and their use

(57) Alkalophilic and thermophilic cellulases having high stability to elevated temperatures and pH have been isolated from an organism of unknown species, which most closely resembles those in the *Caldicellulosiruptor* genus and which has been called by us, Tok7B.1, These cellulases have been cloned and expressed in a recombinant system, so that they can be produced in quantity. These are particularly useful in treating cellulosic materials including cotton-containing fabrics, as detergent additives. and in aqueous compositions. We also provide genomic DNA which can be used in recombinant expression vectors and expression systems to produce enhanced alkali and/or temperature stability properties in cellulases other than those specifically described.

Figure 2.

Blast sequence homology search with the identified N-terminal peptides shows the proteins have homology with Families 9 & 10 from Glycosyl hydrolases. Areas of homology between sequenced N-termini are shown in black backgrounds with white lettering.

Peptide No.	Amino-terminal amino acid sequence	Glycosyl Hydrolase Family basec on amino acid homology comparisons
B1	AAYNYGEALQKAIMFYEFXM	
B3	AAYNYGEALQ	Glycosyl hydrolase
B5	GAYNYGEALQ	Family 9
B6	GAYNY	
B2	APDWSIPSLWESKYND	Glycosyl hydrolase
B4	APGWSIPSLW	Family 10

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Description

A FIELD OF THE INVENTION

[0001] The present invention is directed to improved methods for treating cellulosic materials, including cotton-containing fabrics and non-cotton containing cellulose fabrics with novel truncated cellulase enzymes. In addition, this invention relates to novel truncated cellulase enzymes which exhibit cellulase activity, DNA constructs encoding the enzymes, cellulolytic agents comprising the enzymes, and detergent and water purifying or conditioning compositions containing the enzymes. In particular, this invention provides thermophilic cellulases isolated from a thermophilic anaerobic bacterial strain found in New Zealand. The cellulase genes from this organism are identified and sequenced, and the cellulases expressed from this bacterium are shown to be particularly useful in the abrasion of denim, and in the manufacture of clothing having a "stone wash" look. Most importantly, the cellulases of this invention possess unexpected proteolytic and chemical stability, as well as thermal and pH stability in hot alkaline solutions, thereby rendering them important to as laundry detergent additives in many industrial and home washing applications.

B BACKGROUND OF THE INVENTION

[0002] During or shortly after their manufacture, cotton-containing fabrics can be treated with cellulase enzymes in order to impart desirable properties to the fabric. For example, in the textile industry, cellulase has been used to improve the feel and/or appearance of cotton-containing fabrics, to remove surface fibers from cotton-containing knits, for imparting a "stone washed appearance to cotton-containing denims and the like.

[0003] Clothing made from cellulose fabric, such as cotton denim, is stiff in texture due to the presence of sizing compositions used to ease manufacturing, handling and assembling of clothing items. It typically has a fresh dark dyed appearance. One desirable characteristic of indigo-dyed cloth is the alteration of dyed threads with white threads, which give denim a white on blue appearance.

[0004] After a period of extended wear and laundering, the clothing items, particularly denim, can develop in the clothing panels and on the seams, localized areas of variation in the form of a lightening, in the depth and density of color. In addition, a general fading of the clothes, some pucker in the seams and some wrinkling in the fabric panels can often appear. Additionally, after laundering, sizing is substantially removed from the fabric resulting in a softer feel. In recent years such a distressed or "stonewashed" look, particularly in denim clothing has become very desirable to a substantial proportion of the public.

[0005] Previous methods for producing the distressed look included stonewashing of a clothing item or items in a large tub with pumice stones having a particle size of about 1 by 1 inches and with smaller pumice particles generated by the abrasive nature of the process. Typically the clothing item is tumbled with the pumice while wet for a sufficient period such that the pumice abrades the fabric to produce in the fabric panels, localized abraded areas of lighter color and similar lightened areas in the seams. Additionally the pumice softens the fabric and produces a fuzzy surface similar to that produced by the extended wear and laundering of the fabric. This method also enhances the desired white on blue contrast described above.

[0006] The use of pumice stones has several disadvantages, including overload damage to the machine motors, mechanical damage to transport mechanisms and washing drums, environmental waste problems from the grit produced and high labor costs associated with the manual removal of the stones from the pockets of the garments.

[0007] In view of the problems associated with pumice stones in stonewashing, cellulase solutions are used as a replacement for the pumice stones under agitating and cascading conditions, i. e., in a rotary drum washing machine, to impart a "stonewashed" appearance to the denim.

[0008] Cellulases are enzymes which hydrolyze cellulose (β-1,4-D-glucan linkages) and produce as primary products glucose, cellobiose, cello-oligosaccharides and the like. Cellulases are produced by a number of microorganisms and comprise several different enzyme classifications including those identified as exo-cellobiohydrolases (CBH), endoglucanases (EG), and β-glucosidases (BG). Enzymes within these classifications can be separated into individual components. The complete cellulase system comprising CBH, EG, and BG components synergistically act to convert crystalline cellulose to glucose.

[0009] A problem with the use of complete cellulase compositions from previously described microorganism sources for stonewashing dyed denim is the incomplete removal of colorant caused by redeposition or "backstaining" of some of the dye back onto the cloth during the stonewashing process. In the case of denim fabric, this causes recoloration of the blue threads and blue coloration of the white threads, resulting in less contrast between the blue and white threads and abrasion points (i.e., a blue on blue look rather than the preferred white on blue). This redeposition is objectionable to some users.

[0010] Some cellulases are used commercially even though they result in backstaining because of their higher activity in denim material. Either high specific activity or a high level of purity results in a higher degree of abrasion in a signif-

icantly shorter processing time and therefore is preferable to commercial denim processors.

[0011] Attempts to reduce the amount of redeposition of dye included the addition of extra chemicals or enzymes, such as surfactants, proteases, or other agents, into the cellulase wash to help disperse the loosened dye. In addition, processors have used less active whole cellulase, along with extra washings. However this results in additional chemical costs and longer processing times. Finally the use of enzymes and stones together leave the processor with all the problems caused by the use of the stones alone. Accordingly, it would be desirable to find a method to prevent redeposition of colorant during stonewashing with cellulases.

[0012] There have been previous attempts to prevent backstaining. Patent WO 92/06221 of Genencor pertains to backstaining and indicates that the cellulose biohydralase (CBH) found in fungal cellulases is largely responsible for strength loss of the fabric and that a 5 to 1 ratio of endoglucanase to CBH is desirable. WO 96/23928, also to Genencor, relates to use of a truncated cellulase core enzyme. Both of these references emphasize the use of buffers to stabilize the cellulase solution in the wash environment. In the art it is recognized that cellulase activity is pH dependent. Most cellulases will exhibit cellulolytic activity within an acidic to neutral pH range, and the pH of an unbuffered cellulase solution could be outside the range required for cellulolytic activity. This can be undesirable and requires the addition of reagents to lower the pH of the denim following the wash cycle increasing the processing expense.

[0013] Applications of cellulases for textile processing and in commercial detergents demand proteins which are stable under highly alkaline conditions in the presence of surfactants as well as elevated temperatures.

C BRIEF DESCRIPTION OF THE INVENTION

[0014] Microorganisms from New Zealand hot springs are a recognized potential source of alkalophilic and thermophilic enzymes. We have examined numerous of these microorganisms isolated from thermal pools for their cellulase activity under alkaline conditions. The approach used was to grow the isolated bacterial cultures on cotton in order to enrich for strains that contain cellulase activity. Selected strains were grown on a larger scale and culture supernatants were then individually screened for the desired stone-wash effect. A particular strain of unknown species, but most closely resembles those in the *Caldicellulosiruptor* genus and which has been called by us, Tok7B.1, was identified from this testing. Further investigation resulted in the discovery, in accord with this invention, of six different glycosidase containing genes, designated A through F, which were identified and sequenced. These genes, or gene fragments, were selected for cellulase activity, cloned and expressed. The expressed proteins, especially those designated E1, E1/2, B5, B4/5, and E3/B5 were purified and characterized. These enzymes were shown to have alkaline activity profiles with maximal activity near pH 8.0. These proteins were tested in the textile processing applications including stone washing, and anti-staining or anti-graying, as well as other applications using alkaline pH and/or elevated temperatures, and demonstrated excellent properties in these applications. These highly active cellulase proteins, the DNA encoding these cellulase genes, and recombinant production methods and means for such production of the highly active cellulases are all provided by the invention.

[0015] This invention demonstrates that intact gene products are not required or necessarily desirable for use in many textile processing applications, and that the stability and functionality of these proteins can be varied dramatically by selective combination different genetic fragments, thereby enhancing the activity of the novel proteins herein claimed. The stability enhancing gene fragments can also be expressed with other cellulase genes to confer the improved thermal or high alkaline stability on previously described cellulase proteins.

D SUMMARY OF THE INVENTION

[0016] This invention describes thermophilic bacterial genes that encode multidomain genes containing combinations of cellulase, xylanase or cellobiohydralase activities. Truncated forms of these genes have demonstrated useful stonewash and detergent application activities with cotton cloth. Specific oligonucleotide sequences were identified that when used as PCR primers were shown to amplify genetic sequences that, encode a series of protein domains containing glycohyrolase, thermal stabilizing and cellulose binding activities. A specific protein domain designated CelE2 was shown to function as a thermal stabilizing domain. The addition of this domain to an endoglucanase increased the thermostability by 25C. This activity could be widely applicable for enhancing the thermal stability of other genes.

[0017] The genes were obtained from the thermophilic obligate anaerobic bacterium by PCR amplification of the genomic DNA. The synthetic oligonucleotide primer sequences used for the gene amplification reactions were based on either N-terminal protein sequence data, from which degenerate probes were designed, or from genomic expression library constructs that had been screened for cellulase, cellolobiosidase or xylanase activities. These specific oligonucleotide probes can serve to amplify genes useful in stone washing and/or detergent applications from other unknown bacteria that have cellulase genes.

[0018] Encoded gene fragments from the amplified genes identified as having cellulase activity were expressed in

E. coli either singly or in combination with cellulose binding domains and /or thermal stabilizing domains. The expressed proteins were and purified to homogeneity and characterized. Cotton containing cloth treated with certain of these truncated gene constructs having endoglucanase domains and/or cellulose binding domains gave a stonewash appearance, and with other endoglucanase constructs a soil antiredeposition effect.

E BRIEF DESCRIPTION OF DRAWINGS

[0019] Figures 1A and 1B are a composite drawing of protein bands containing cellulase activity purified from the supernatant broth of the Tok7B.1 organism, and their N-terminal sequences.

[0020] Figure 2 shows the results of the BLAST sequence homology search with the sequenced protein N-termini.

[0021] Figure 3 is a diagram of two consensus primers TokcelA and TokcelB and their relationship to other family 9 cellulases.

[0022] Figure 4A and 4B show the genomic walking primers and the regions amplified to obtain the complete *celE* gene and flanking regions. Figure 4C depicts a restriction map and the genetic domain structure of the *celE* gene sequence, including flanking upstream and downstream sequences.

[0023] Figure 5A is a map of W2-4 and N-17 genomic DNA fragments isolated from the Tok7B.1 genome that express cellulase activity. Figure 5B depicts the genomic walking primers and the regions amplified to obtain the complete *celA* and *celB* genes. The genetic domain structure and restriction map of *celA* and *celB* is shown in Figure 5C.

[0024] Figure 6 is a complete summary of the genetic domain structure of *celA*, *celB* and *celE* genes.

[0025] Figures 7a and 7b are a map of the restriction sites and domain structure of the Tok7B.1 genes *celC, celD, celE, celF, celG* and *celH* genes. Also the genomic walking primers used to amplify and identify each of these genes and the genetic regions amplified are indicated.

[0026] Figure 8 is a diagram of the genes and gene fragments transferred into pJLA602 controlled expression plasmid vectors.

[0027] Figure 9 is a phylogenetic analysis of the Tok7B.1 organism.

[0028] Figures 10-12 are flow diagrams for construction of the expression plasmids of pMcelE-1 and pMcelE1-2.

[0029] Figure 13 is a flow diagram for construction of the expression plasmid pMcelE1-2-3.

[0030] Figure 14 is a flow diagram for construction of the expression plasmid of pcelB4-5.

[0031] Figure 14A is a flow diagram for construction of the expression plasmid of pcelE3/B5.

[0032] Figure 15 shows the sequence analysis and MALDI-TOF of the expressed cellulases.

[0033] TABLE I lists the oligonucleotide primers designed and synthesized for study of the cellulase genes in the Tok7B.1 organism.

[0034] TABLE II lists the oligonucleotides designed for PCR amplification and directional ligation of the Tok7B.1 genes into controlled expression vectors.

[0035] TABLE III shows the gene constructs expressed in E. coli by a T-7 promoter.

[0036] TABLE IV is a summary T-7 expressed cellulases, their pH rate profiles, thermal stabilities and effectiveness in the stonewash application.

F DETAILED DESCRIPTION OF THE INVENTION

I DEFINITIONS

[0037] "Cotton-containing fabric" means sewn or unsewn fabrics made of pure cotton or cotton blends including cotton woven fabrics, cotton knits, cotton denims, cotton yarns and the like. When cotton blends are employed, the amount of cotton in the fabric should be at least about 40 percent by weight cotton; preferably, more than about 60 percent by weight cotton; and most preferably, more than about 75 percent by weight cotton. When employed as blends, the companion material employed in the fabric can include one or more non-cotton fibers including synthetic fibers such as polyamide fibers (for example, nylon 6 and nylon 66), acrylic fibers (for example, polyacrylonitrile fibers), and polyester fibers (for example, polyethylene terephthalate), polyvinyl alcohol fibers (for example, Vinylon), polyvinyl chloride fibers, polyvinylidene chloride fibers, polyurethane fibers, polyurea fibers and aramide fibers.

[0038] "Cellulose containing fabric" means any cotton or non-cotton containing cellulosic fabric or cotton or non-cotton containing cellulose blend including natural cellulosics and manmade cellulosics (such as Jute, flax, ramie, rayon, and the like). Included under the heading of manmade cellulose containing fabrics are regenerated fabrics that are well known in the art such as rayon. Other manmade cellulose containing fabrics include chemically modified cellulose fibers (e g., cellulose derivatized by acetate) and solvent-spun cellulose fibers (e.g. lyocell). Of course, included within the definition of cellulose containing fabric is any garment or yarn made of such materials. Similarly, "cellulose containing fabric" includes textile fibers made of such materials.

[0039] "Treating composition" means a composition comprising a truncated cellulase component which may be used

in treating a cellulose containing fabric. Such treating includes, but is not limited to, stonewashing, modifying the texture, feel and/or appearance of cellulose containing fabrics or other techniques used during manufacturing of cellulose containing fabrics. Additionally, treating within the context of this invention contemplates the removal of "dead cotton", from cellulosic fabric or fibers. i.e. immature cotton which is significantly more amorphous than mature cotton. Dead cotton is known to cause uneven dyeing. Additionally, "treating composition" means a composition comprising a truncated cellulase component which may be used in washing of a soiled manufactured cellulose containing fabric. For example, truncated cellulase may be used in a detergent composition of, washing laundry. Detergent compositions useful in accordance with the present invention include special formulations such as pre-wash, pre-soak and home-use color restoration compositions. Treating compositions may be in the form of a concentrate which requires dilution or in the form of a dilute solution or form which can be applied directly to the cellulose containing fabric.

[0040] It is Applicants' present belief that the action pattern of cellulase upon cellulose containing fabrics does not differ significantly whether used as a stonewashing composition during manufacturing or during laundering of a soiled manufactured cellulose containing fabric. Thus, improved properties such as abrasion, redeposition of dye, strength loss and improved feel conferred by a certain cellulase or mixture of cellulases are obtained in both detergent and manufacturing processes incorporating cellulase. Of course, the formulations of specific compositions for the various textile applications of cellulase, e.g., stonewashing or laundry detergent or pre-soak, may differ due to the different applications to which the respective compositions are directed, as indicated herein. However, the improvements effected by the addition of cellulase compositions will be generally consistent through each of the various textile applications.

II PREPARATION OF TRUNCATED CELLULASE ENZYMES

[0041] The present invention relates to the use of truncated cellulases and derivatives of truncated cellulases. These enzymes are preferably prepared by recombinant methods. Additionally, truncated cellulase proteins for use in the present invention may be obtained by other art recognized means such as chemical cleavage or proteolysis of complete cellulase protein.

[0042] The invention provides recombinant cellulase proteins which are alkalophilic and thermophilic and highly active and useful in washing applications, or in any applications including textile processing in which it is desirable to break down cellulose or cellulosic materials. It further provides DNA, free from its native genomic source, which encodes the recombinant cellulase active proteins in accord with the invention. In another preferred embodiment of this invention, we also provide genomic DNA which can be used in recombinant expression vectors and expression systems to produce enhanced alkali and/or temperature stability properties in cellulases other than those specifically described.

[0043] Also provided by the invention are bacteria cells capable of producing a native cellulase in accord with the invention and from which DNA encoding cellulases in accord with the invention may be obtained. Also provided is the native cellulase purified with respect to its native origins and associated native proteins such as by having a high protein purity or even absolute purity of at least 50%, e.g. 75%.

[0044] By way of specific preferred embodiments, this invention provides the following five particularly highly active cellulase proteins: E1, E1/2, B4/5, B5, and E3/B5.

[0045] E1 has an amino acid sequence of 446 amino acids extending from amino acid position No Y39 through amino acid position No D481 as given in Seq. ID No 44, or a function equivalent analogue thereof. DNA encoding this cellulase may vary in accord with the genetic code and a specific embodiment of such a DNA sequence comprises the DNA extending from nucleotide position No 748 through nucleotide position No 2076 as given in Sequence ID No 2.

[0046] E1/2 has an amino acid sequence of 600 amino acids extending from amino acid position No Y 39 through amino acid position No G635 as given in Seq. ID No 44, or a function equivalent analogue thereof. DNA encoding this cellulase may vary in accord with the genetic code and a specific embodiment of such a DNA sequence comprises the DNA extending from nucleotide position No 748 through nucleotide position No 2538 as given in Sequence ID No 2.

[0047] B4/5 has an amino acid sequence of 645 amino acids extending from amino acid position No K635 through amino acid position No N 1426 as given in Seq. ID No 43, or a function equivalent analogue thereof. DNA encoding this cellulase may vary in accord with the genetic code and a specific embodiment of such a DNA sequence comprises the DNA extending from nucleotide position No 8601 through nucleotide position No 10532 as given in Sequence ID No 1.

[0048] B/5 has an amino acid sequence of 418 amino acids extending from amino acid position No A 1001 through amino acid position No P 1424 as given in Seq. ID No 43, or a function equivalent analogue thereof. The B-5 protein can also end at K 1425 or N 1426, to include 419 or 420 amino acids, respectively. DNA encoding this cellulase may vary in accord with the genetic code and a specific embodiment of such a DNA sequence comprises the DNA extending from nucleotide position No 9255 through nucleotide position No 10526 as given in Sequence ID No 1.

[0049] E3/B5 has an amino acid sequence of 616 amino acids, and is a hybrid protein formed from sequences taken from the E and the B portions of the native sequences. The cel B sequence is that described from amino acid position

No K635 through amino acid position No N 1426 as given in Seq. ID No 43. DNA encoding this hybrid cellulase may vary in accord with the genetic code, but a specific embodiment of such a DNA sequence comprises the DNA starting from the celE gene at G2659. ending at G3123, as given in Sequence ID No 2; then joined to a segment taken from the celB gene starting at G9153 and ending at A10.532., as given in Sequence ID No 1., or functional equivalent analogues thereof. This E3/B5 protein and its nucleotide sequence are described in Seq ID Nos 46 and 47 respectively.

[0050] As will be recognized by those skilled in the art, DNA encoding active cellulases in accord with the invention may be modified in various ways to produce such cellulases for practical usage. For example, the DNA encoding a signal sequence may be removed and replaced by the codon ATG encoding for Met at amino acid position No. 31, using known techniques. The resulting DNA which lacks a signal sequence may be used to express active cellulase in accord with the invention, more particularly in *E. coli*, which cellulase product depending on the host strain will produce a cellulase with or without Met at its N-terminus, or mixtures of such products. Similarly, the signal sequence may be replaced by known techniques with other signal sequences to improve production, particularly secretion into the production media, and/or to adapt the DNA to particular hosts for production.

[0051] The cellulase gene-containing inserts cloned and provided in accord with our invention contain all the control or regulatory sequences necessary for expression of the structural gene in bacterial hosts, particularly Bacillus and *E. coli* hosts. These sequences, such as promoter sequences, ribosome binding site sequences and the like may also be modified or replaced in whole or in part by other control sequences using known techniques to improve production and/or to adapt the DNA to particular hosts for production. When such a change is made, the resulting DNA sequence is deemed to involve the structural gene in sequence with heterologous DNA.

[0052] The DNA encoding an active alkalophilic and thermophilic cellulase in accord with the invention may be incorporated into a wide variety of vectors for various purposes such as replication of such DNA or expression of the structural gene or for purposes of causing incorporation of the DNA into the genome of a host cell for ultimate expression of the encoded gene. Such vectors will typically involve DNA sequences containing the DNA encoding the active cellulase recombined with other heterologous DNA. The terms heterologous DNA and the like as used herein generally refer to a DNA sequence which has a functional purpose and which is either different from the sequences in or obtained from a source other than the native Tok7B.1 DNA from which the instant gene was cloned, thereby creating a continuous sequence which is not found or associated with the cellulase gene in the native Tok7B.1 source. Examples of such functional sequences are many and include for purposes of illustration origins of replications, genes for antibiotic resistance and also various control sequences, such promoter sequences to be used for effecting expression of the structural gene itself, as well as flanking sequences suitable for causing insertion of DNA containing the gene coding sequence into a host genome. Such vectors include for illustration only those commonly referred to plasmids and those which are viral vectors. The construction of vectors is well-known and DNA sequences of widely different origins and/or recombinations are available for such construction, such sequences also commonly called plasmids, viral vectors and the like. For example, a vector in accord with the invention and used by us can be obtained from the known plasmid pUC18 which contains the pBR 322-derived ampicillin resistance gene and origin of replication, together with a portion of the *E. coli* lacZ gene (lacZ') encoding the a-complementation peptide.

[0053] This lacZ' fragment has been engineered to contain a multiple cloning site (MCS). DNA inserted into the MCS inactivates the lacZ' gene, providing blue/white color selection of recombinants when appropriate hosts and indicator plates are used. The complete gene or clone we obtained can be inserted or ligated into the MCS and expressed in an *E. coli* host by operation of its own native control sequences.

[0054] In general, the vectors of the invention are constructed with reference to suitability for incorporation into particular host cells, and such transformed cells are also a part of the invention. As used herein, the term "transformed" and the like means the incorporation of vector DNA into a host cell independent of the purpose in terms of replication of the recombinant gene or its expression, or both, and whether the vector DNA remains intact in the cell or its contained cellulase encoding gene is incorporated for expression into the cell genome. The vectors of the invention may be transformed into any of a variety of cell types such as bacterial cell, yeast cells, insect and mammalian cells. Preferably, the transformed cells are bacteria or yeast cells, and more preferably are gram negative bacteria such as *E. coli* or gram positive bacteria such as Streptomyces or Bacillus cells where such Bacillus cells are not of thermophilic source, such preferred Bacillus types including Bacillus subtilis and the like. Methods for transforming cells with vectors are generally well-known.

[0055] The invention also provides a process for producing the recombinant cellulase active proteins of the invention comprising culturing cells transformed with a recombinant expression vector of the invention comprising promoter DNA operatively controlling expression of the DNA encoding the cellulase protein. Methods of culturing such transformed cells to effect their multiplication and expression of the cellulase encoding gene of the transformed vector DNA are also well-known. Procedures for recovery of the recombinantly produced proteins are also known and may be used to obtain the cellulase of the invention in the more practical forms for use. In general, the recombinantly produced cellulase as expressed by the transformed cells may be retained within the cells and/or secreted into the culture media. When retained in quantity within the cells, the cells are lysed such as in a Warring Blender, sonifier or pressure cell to liberate

the cellulase into the culture media which is then usually treated to separate cellular debris and preferably filtered to obtain the cellulase in the resulting aqueous supernatant or filtrate. When secreted into the media, the culture liquid media or supernatant containing the cellulase is simply separated from the cells. Such filtrates and supernatants may then be used as a basis for a product for treatment of cellulosic materials, typically after concentration. Such cellulase-containing liquids may also be treated, for example by microfiltration, to separate undesired materials including lower molecular weight proteins. The resulting aqueous cellulase-containing compositions may also be treated to enhance their storage or use properties, for example, by addition of buffers to enhance stability of the cellulase. Hence, the cellulase products may be buffered between pH 5 to 10, preferably pH 7 to 9, using, for example, Tris buffer.

[0056] The cellulases of the present invention have been found to be particularly useful for additives used in the cleaning or treatment of cellulose fabrics, including cotton-containing fabrics. They exhibit high activity even at high temperatures or high pH, thereby facilitating their suitability of aqueous detergent solutions and formulations.

[0057] It will be recognized that the cellulases of the invention are obtained from a microorganism characteristic of those which are thermophilic and alkalophilic and which produce a variety of enzymes which may be similarly classified by favoring conditions encountered in natural thermally heated alkaline pools. A variety of microorganisms have been identified in such pools. The cellulases of this invention originate from a particular strain of unknown species which most closely resembles those in the *Caldicellulosiruptor* genus and which has been called by us, Tok7B.1.

III DEPOSITS

[0058] We have under the Budapest Treaty conditions, deposited with the American Type Culture Collection at Rockville, MD, USA, a biologically pure culture of the cells indicated below, which deposits were assigned the Accession Numbers given below along with their date of deposit.

Identification and Content of Deposit	Accession No.	Deposit Date
E. coli BL21 (DE3) Cel E	ATCC 98523	August 29, 1997
E. coli DH α F' 1Q Cel B	ATCC 98524	August 29, 1997
Tok7B.1 bacterial strain	ATCC 202028	September 10, 1997

[0059] As will be recognized, any of the above deposits may be cultured under condition to cause expression of a cellulase of the invention in accord with the experiments described herein and such cellulase products recovered in a variety of product forms for use as also described herein. Alternatively, cultures of the deposited cells may be grown to multiple the number of copies of their contained plasmidal clones and the cellulase gene and coding sequence may be separated from the plasmids by the use of restriction enzymes, preferably by partial digest with Sau3AI, and the DNA encoding the cellulase (for example, an approx. 1.57 Kb fragment upon Sau3AI partially digest) used for a variety of purposes including production of active cellulase protein of the invention in a wide variety of other expression systems.

IV METHODS OF TREATING CELLULOSE CONTAINING FABRIC USING TRUNCATED CELLULASE ENZYMES

[0060] As noted above, the present invention pertains to methods for treating cellulose containing fabrics with a truncated cellulase enzyme. The use of the truncated cellulase composition of this invention provides the novel and surprising result of effecting a relatively low level of dye redeposition while maintaining an equivalent level of abrasion compared to prior art cellulase treatment. Because the level of abrasion acts as an indicator of the quality and effectiveness a of particular cellulase treatment techniques, e.g., stonewashing or laundering, the use of the instant invention provides a surprisingly high quality textile treatment composition. In the laundering context, abrasion is sometimes referred to as color clarification, defuzzing or biopolishing.

[0061] The present invention specifically contemplates the use of truncated cellulase core, alone or in combination with additional cellulase components, to achieve excellent abrasion with reduced redeposition when compared to non-truncated cellulase. Additionally, naturally occurring cellulase enzymes which lack a binding domain are contemplated as within the scope of the invention. It is also contemplated that the methods of this invention will provide additional enhancements to treated cellulose containing fabric, including improvement in the feel and/or appearance of the fabric.

A) METHODOLOGY FOR STONEWASHING WITH TRUNCATED CELLULASE COMPOSITIONS

[0062] According to one aspect of the present invention, the truncated cellulase compositions described above may be employed as a stonewashing composition. Preferably, the stonewashing composition of the instant invention com-

prises an aqueous solution which contain a an effective amount of a truncated cellulase together with other optional ingredients including, for example, a buffer, a surfactant, and a scouring agent.

[0063] An effective amount of truncated cellulase enzyme composition is a concentration of truncated cellulase enzyme sufficient for its intended purpose. Thus an "effective amount" of truncated cellulase in the stonewashing composition according to the present invention is that amount which will provide the desired treatment. e.g., stonewashing. The amount of truncated cellulase employed is also dependent on the equipment employed, the process parameters employed (the temperature of the truncated cellulase treatment solution, the exposure time to the cellulase solution, and the like), and the cellulase activity (e.g., a particular solution will require a lower concentration of cellulase where a more active cellulase composition is used as compared to a less active cellulase composition). The exact concentration of truncated cellulase can be readily determined by the skilled artisan based on the above factors as well as the desired result. Preferably the truncated cellulase composition is present in a concentration of from 1-1000 PPM, more preferably 10-400 PPM and most preferably 20-100 PPM total protein.

[0064] Optionally, a buffer is employed in the stonewashing composition such that the concentration of buffer is that which is sufficient to maintain the pH of the solution within the range wherein the employed truncated cellulase exhibits activity which, in turn, depends on the nature of the truncated cellulase employed. The exact concentration of buffer employed will depend on several factors which the skilled artisan can readily take into account. For example, in a preferred embodiment, the buffer as well as the buffer concentration are selected so as to maintain the pH of the final truncated cellulase solution within the pH range required for optimal cellulase activity. Preferably, buffer concentration in the stonewashing composition is about 0.001N or greater. Suitable buffers include, for example, citrate and acetate.

[0065] In addition to truncated cellulase and a buffer, the stonewashing composition may optionally contain a surfactant. Preferably, the surfactant is present in a concentration in the diluted wash mediums of greater than 100 PPM, preferably from about 200-15.000 PPM. Suitable surfactants include any surfactant compatible with the cellulase and the fabric including, for example, anionic, non-ionic and ampholytic surfactants. Suitable anionic surfactants for use herein include linear or branched alkylbenzenesulfonates; alkyl or alkenyl ether sulfates having linear or branched alkyl groups or alkenyl groups; alkyl or alkenyl sulfates; olefinsulfonates; alkanesulfonates and the like. Suitable counter ions for anionic surfactants include alkali metal ions such as sodium and potassium; alkaline earth metal ions such as calcium and magnesium; ammonium ion; and alkanolamines having 1 to 3 alkanol groups of carbon number 2 or 3. Ampholytic surfactants include quaternary ammonium salt sulfonates, and betaine-type ampholytic surfactants. Such ampholytic surfactants have both the positive and negative charged groups in the same molecule. Nonionic surfactants generally comprise polyoxyalkylene ethers, as well as higher fatty acid alkanolamides or alkylene oxide adduct thereof, and fatty acid glycerine monoesters. Mixtures of surfactants can also be employed in manners known in the art.

[0066] In a preferred embodiment, a concentrated stonewashing composition can be prepared for use in the methods described herein. Such concentrates would contain concentrated amounts of the truncated cellulase composition described above, buffer and surfactant, preferably in an aqueous solution. When so formulated, the stonewashing concentrate can readily be diluted with water so as to quickly and accurately prepare stonewashing compositions according to the present invention and having the requisite concentration of these additives.

[0067] Preferably, such concentrates will comprise from about 0.1 to about 50 weight percent of a cellulase composition described above (protein); from about 0.1 to about 80 weight percent buffer; from about 0 to about 50 weight percent surfactant, with the balance being water. When aqueous concentrates are formulated, these concentrates can be diluted so as to arrive at the requisite concentration of the components in the truncated cellulase solution as indicated above. As is readily apparent, such stonewashing concentrates will permit facile formulation of the truncated cellulase solutions as well as permit feasible transportation of the concentration to the location where it will be used. The stonewashing concentrate can be in any art recognized form, for example, liquid, emulsion, gel, or paste. Such forms are well known to the skilled artisan.

[0068] Other materials can also be used with or placed in the stonewashing composition of the present invention as desired, including stones, pumice, fillers, solvents, enzyme activators, and other anti-redeposition agents.

[0069] The cellulose containing fabric is contacted with the stonewashing composition containing an effective amount of the truncated cellulase enzyme or derivative by intermingling the treating composition with the stonewashing composition, and thus bringing the truncated cellulase enzyme into proximity with the fabric. For example, if the treating composition is an aqueous solution, the fabric may be directly soaked in the solution. Similarly, where the stonewashing composition is a concentrate, the concentrate is diluted into a water bath with the cellulose containing fabric. When the stonewashing composition is in a solid form, for example a pre-wash gel or solid stick, the stonewashing composition may be contacted by directly applying the composition to the fabric or to the wash liquor.

[0070] The cellulose containing fabric is incubated with the stonewashing solution under conditions effective to allow the enzymatic action to confer a stonewashed appearance to the cellulose containing fabric. For example, during stonewashing, the pH, liquor ratio, temperature and reaction time may be adjusted to optimize the conditions under which the stonewashing composition acts. "Effective conditions" necessarily refers to the pH, liquor ratio, and temperature which allow the truncated cellulase enzyme to react efficiently with cellulose containing fabric. The reaction con-

ditions for truncated cellulase core, and thus the conditions effective for the stonewashing compositions of the present invention, are substantially similar to well known methods used with corresponding non-truncated cellulases. Similarly, where a mixture of truncated and non-truncated cellulase is utilized, the conditions should be optimized similar to where a similar combination may have been used. Accordingly, it is within the skill of those in the art to maximize conditions for using the stonewashing compositions according to the present invention.

[0071] The liquor ratios during stonewashing, i.e., the ratio of weight of stonewashing composition solution (i.e., the wash liquor) to the weight of fabric, employed herein is generally an amount sufficient to achieve the desired stonewashing effect in the denim fabric and is dependent upon the process used. Preferably, the liquor ratios are from about 4:1 to about 50:1; more preferably from 5:1 to about 20:1, and most preferably from about 10:1 to about 15:1. Reaction temperatures during stonewashing with the present stonewashing compositions are governed by two competing factors. Firstly, higher temperatures generally correspond to enhanced reaction kinetics, i.e., faster reactions, which permit reduced reaction times as compared to reaction times required at lower temperatures. Accordingly, reaction temperatures are generally at least about 10°C and greater. Secondly, cellulase is a protein which loses activity beyond a given reaction temperature which temperature is dependent on the nature of the cellulase used. Thus, if the reaction temperature is permitted to go too high, then the cellulolytic activity is lost as a result of the denaturing of the cellulase As a result, the maximum reaction temperatures employed herein are generally about 65°C. In view of the above, reaction temperatures are generally from about 30°C to about 65°C; preferably, from about 35°C to about 60°C; and more preferably, from about 35°C to about 55°C.

[0072] Reaction times are dependent on the specific conditions under which the stonewashing occurs. For example, pH, temperature and concentration of truncated cellulase will all effect the optimal reaction time. Generally, reaction times are from about 5 minutes to about 5 hours, and preferably from about 10 minutes to about 3 hours and, more preferably, from about 20 minutes to about 1 hour.

[0073] Cellulose containing fabrics treated in the stonewashing methods described above using truncated cellulase compositions according to the present invention show reduced redeposition of dye as compared to the same cellulose containing fabrics treated in the same manner with an non-truncated cellulase composition.

B) METHODOLOGY FOR TREATING CELLULOSE CONTAINING FABRICS WITH A DETERGENT COMPOSITION COMPRISING TRUNCATED CELLULASE ENZYME

[0074] According to the present invention, the truncated cellulase composition described above may be employed in detergent compositions. The detergent compositions according to the present invention are useful as pre-wash compositions, pre-soak compositions, or for detergent cleaning during the regular wash cycle. Preferably, the detergent composition which can be dry mixed or in an aqueous liquid formulation, of the present invention comprises an effective amount of truncated cellulase, and a surfactant, and optionally include other ingredients and additives commonly employed in detergent formulations. An effective amount of truncated cellulase employed in the detergent compositions of this invention is an amount sufficient to impart improved anti-graying, anti-staining, anti-backstaining, or anti-soil deposition of cotton or cellulosic containing fabrics. Preferably, the truncated cellulase employed is in a concentration of about 0.001% to about 25%, more preferably, about 0.02% to about 10% by weight percent of detergent.

[0075] The specific concentration of truncated cellulase enzyme employed in the detergent composition is preferably selected so that upon dilution into a wash medium, the concentration of truncated cellulase enzyme is in a range of about 0.1 to about 1000 PPM, preferably from about 0.2 PPM to about 500 PPM, and most preferably from about 0.5 PPM to about 250 PPM total protein. Thus, the specific amount of truncated cellulase enzyme employed in the detergent composition will depend on the extent to which the detergent will be diluted upon addition to water so as to form a wash solution.

[0076] At lower concentrations of truncated cellulase enzyme, i.e., concentrations of truncated enzyme lower than 20 PPM, the decreased backstaining or redeposition with equivalent surface fiber abrasion when compared to prior art compositions will become evident after repeated washings. At higher concentrations, i.e., concentrations of truncated cellulase enzymes of greater than 40 PPM, the decreased backstaining with equivalent surface fiber removal will become evident after a single wash.

[0077] This invention is illustrated by the following procedures and examples.

[0078] Applications of cellulases for textile processing and in commercial detergents demand proteins that are stable under conditions of alkaline pH and elevated temperatures.

V EXAMPLES

Isolation of cellulase secreting microorganisms from alkaline thermal pools

[0079] To identify thermal stable glycolytic proteins, microorganisms were isolated from the water and sediment

samples taken from geothermal pools in the central volcanic region of New Zealand's North Island. The criteria for the pools sampled were temperatures of at least 50° C and pH values of greater than 6.0. A total of twenty samples were collected from geothermal pools that met the criteria. Each of the samples contained a complex mixture of microorganisms. In order to enrich the samples for microorganisms that expressed cellulase genes with desired cellulase activity 1 mL volumes of the collected sample were inoculated into 10 mL of 2/1 medium in Hungate tubes containing either amorphous cellulose (7g/L) or unbleached cotton fabric (approximately 1 cm square) as cellulose substrate, at pH 7.0 and pH 8.5. These tubes were incubated at 70° C and the cultures viewed microscopically after 4 days. The enrichment strategy was based on the assumption that the presence of the cellulosic fibers would induce expression of the cellulase genes in the microorganisms, and that those microorganisms would flourish under these conditions. From this collection of organisms the anaerobic, cellulase producer, Tok7B.1 was isolated from a water/sediment sample take from Tokaanu Pool 7, situated in the central volcanic region of the North Island. New Zealand. The pH and temperature of this particular pool at the time of sampling were pH 7.5 and 60° C, respectively.

[0080] The 2/1 medium and amorphous cellulose, pH 7.0 proved the most favorable for the growth of the anaerobic rods from the Tok7B.1 sample, and after further subculturing, PAHBAH (p-hydroxybenzoic acid hydrazide) assays (Lever, 1973) on the concentrated supernatant confirmed the presence of cellulase-producing organisms. The substrate for these PAHBAH assays was 0.2% carboxymethyl cellulose (low viscosity) in 100 mM Taps buffer pH 8.8 at 20° C.

[0081] A pure culture of Tok7B.1 was obtained using a version of the Roll Tube method described by Hungate (1969). Serial dilutions of the positive cultures were make in Hungate tubes containing the growth medium + 18 g/l agar. The agar/culture mixture was solidified around the inside of the sealed tube by rolling in a flat dish containing iced water. Tubes were incubated at 70° C and single colonies removed aseptically using a Pasteur pipette with the tip bent a right angles. A plug of agar was placed in liquid medium and the cells released by crushing against the side of the tube. Positive identification of a cellulase producer was again confirmed by PAHBAH assays of the culture supernatants. To detect secreted cellulases supernatants from the cultures were concentrated approximately ten fold prior to being assayed for cellulase activity using CMCase assay.

[0082] The Tok7B.1 cellulases were identified in a secondary screening assay that served to evaluate the biostone washing effectiveness of the cellulases secreted into the sample supernatant. Each of the cultures selected for screening was fermented in sufficient quantity and the supernatants concentrated in order to provide sufficient activity for the biostone wash testing, approximately 10,000 CMCase units. The supernatants were tested in a 2L drum denim assay at equivalent levels of CMCase activity. The cellulases were tested under the following conditions; pH 7.0 for 60 minutes at 50 ° C using 135g of blue denim samples were washed for 1h at pH 7.0. The light reflectance value on the blue denim cloth and from a swatch of white cloth included in the wash were determined by measuring the level of denim abrasion and backstaining, respectively. Blue denim samples that demonstrated a reflectance value of above 15 and a dose dependent effect with increasing concentrations of fermentation supernatants were considered to contain candidate cellulases. White cloth swatches that have a reflectance of below 4 were acceptable for backstaining. Based on these tests the Tok7B.1 organism was found to produce the most effective cellulases, giving the highest abrasion with the lowest backstaining of the samples tested.

Strategy for identifying industrially useful cellulases

[0083] Our strategy was to identify industrially useful cellulases secreted from the Tok7B.1 organism, then to identify the individual genes responsible for that activity. The following steps were carried out to clone the individual genes, express these genes in an intermediary expression system and test the individual cellulases in the application. The first step in the strategy was to identify the individual proteins secreted by the Tok7B.1 bacterium. Identification of the individual cellulases secreted by the bacterium was important because identification of the genes effective in the application would limit the number of cellulase genes and gene constructs that would have to be expressed and tested.

Cellulase Nomenclature

[0084] Genes and genetic constructs are designated in small letters and are italicized, for example the genes that encode the CelE proteins are designated *celE*. Conversely proteins are designated by capitalizing the first letter and are not italicized, for example, CelE1. The Tok7B.1 cellulase genetic domains are designated in Figure 6, and one should be careful not to confuse these with the protein designations shown in the third column of Table III. For example the CelE1 protein is comprised of the second genetic domain in the *celE* gene.

Identification of N-terminal Sequences of Tok7B.1 cellulases

[0085] The culture supernatant from the Tok7B.1 strain was chromatographed on a Mono-S column (Pharmacia) at

pH 5.0 in 10 mM sodium acetate buffer at a flow rate of 1 ml/min. The bound proteins were eluted with a 30 ml linear gradient of NaCl from 0-250 mM. Each of the fractions collected was assayed for CMCase activity. 73% of the total CMCase activity was collected into fractions and 27% of the activity was found in the column flow-through. The proteins from fractions that demonstrated CMCase activity were electrophoresed on an 8% SDS polyacrylamide gel. Protein bands in fractions containing cellulase activity could be observed in a Coomassie-stained 8% SDS polyacrylamide gel. The cellulase activity of these bands was confirmed in part by overlaying the SDS polyacrylamide gel with an agarose gel containing carboxymethyl cellulose (CMC). Cellulases not denatured by the SDS degrade the CMC in the agarose gel. These areas of degraded CMC can be identified by staining with Congo Red using the methods of Beguin (1983) and Mackenzie and Williams (1984). Proteins of interest were blotted from the SDS-PAGE gel onto an Immobilon membrane and then the amino terminal sequences determined by Edman degradation (Matsudaria, 1987). The sequences determined for each of the individual bands are shown in a composite drawing (Figure 1). CMCase activity that was not captured on the Mono-S column was subsequently buffer-exchanged into 12 mM Tris buffer pH 9.0, chromatographed on a Q sepharose column (1.5 x 6 cm), and eluted with a 30 mL linear gradient of 0-250 mM NaCl. Fractions that contained CMCase activity were electrophoresed on an 8% SDS PAGE and gave a protein band with identical apparent molecular weight and N-terminal sequence to the B5 band (Figure 1) previously identified from the S-sepharose column.

[0086] The N-termini of each of these proteins was determined by Edman degradation. Only two different amino acid sequences were determined from the six proteins N-terminally sequenced. The N-terminus of the *celE* gene product was homologous with four of the proteins identified and the N-terminus of the *celB* gene product wash homologous with the two remaining protein bands. The amino acid sequence information served first to identify the genes that were expressing the cellulases useful for the applications. Second, the N-terminal sequences were compared with the protein sequences in GenBank using the Basic Linear Alignment Search Technique (BLAST. Jauris, et al., 1990). This confirmed that the two proteins sequenced belonged to the glycosyl-hydrolase family. The *celB* gene product has an amino-terminal sequence which shares significant homology with a general class of xylan degrading enzymes referred to as Family F beta-glycanases (Gilkes et al., 1991) or Family 10 glycosyl-hydrolase (Henrisatt, 1991). The CelE gene product shares homology with family E beta-glycanases/Family 9 glycosyl-hydrolases.

Strategy for the cloning of the cellulase genes

[0087] Our strategy for identifying the Tok7B.1 glycolytic genes was to employ two approaches simultaneously. 1) Polymerase chain reaction (PCR) with primers based on the sequence information obtained from the BLAST search was used PCR to amplify gene sequences from the Tok7B.1 genomic DNA preparations. 2) An expression library of the Tok7B.1 genomic DNA was constructed and screened for the expression of proteins able to degrade CMC.

Methods and Prior Art

[0088] Agarose gel electrophoresis, plasmid isolation, M13 mp10 single stranded DNA isolation, use of DNA modifying enzymes and *E. coli* transformation were performed as described by Sambrook et al. (1989).

Genomic DNA Preparation

[0089] Tok7B.1 genomic DNA was prepared from a cell culture which had been grown under anaerobic conditions for 1-2 days without shaking at 70°C in 2/1 media. Cells were harvested from the growth media by centrifugation at 5000 rpm for 10 minutes, then resuspended in 50 ml TES buffer before a second centrifugation step. Cell pellets were then resuspended in 5ml 50mM Tris pH 8.0, mixed with 374µl 0.5M EDTA and incubated for 20 minutes at 37°C. After the addition of 550µl freshly prepared lysozyme (10mg/ml), the mixture was incubated at 70°C for 20 minutes, mixed with 250µl *Streptomyces griseus* protease (40mg/ml) and 310µl 10% SDS, then left to incubate overnight at 70°C. After allowing the lysed cells to cool to room temperature, the resulting clear solution was phenol extracted 2-5 times until no material could be seen to partition at the interface. The remaining volume of the sample was estimated and a 1/10 volume of 3M Sodium acetate was added and mixed, then 2.5 volumes of 95-100% ethanol gently layered onto the top of the sample. DNA could being seen as a stringy white precipitate at the interface of the two liquids and could be removed by spooling onto the end of a Pasteur pipette. Spooled DNA was transferred into a 1.5ml microcentrifuge tube and washed in 70% ethanol before air drying for 1-3 hours. The resulting DNA pellet was resuspended in TE buffer and left overnight to fully dissolve. All genomic DNA preparations were stored at 4°C.

[0090] **Isolation of the Tok7B.1 celE gene using consensus PCR and Genomic walking PCR** The Tok7B.1 *celE* gene, gene product CelE, was identified by amino-terminal sequencing of cellulolytic peptides secreted by Tok7B.1 (Figure 2). The *celE* gene codes for a family 9 glycosyl hydrolase based on comparison to translated gene sequences in the GenBank database. The CelE peptide sequence shared highest similarity to family 9 glycosyl hydrolases from

other thermophilic Clostridial microorganisms. Homology alignments of family 9 genes indicated that it would be possible to design consensus oligonucleotide primers which would bind to DNA coding for clusters of highly conserved amino acids found in all thermophilic Clostridial family 9 glycosyl hydrolases. These consensus primers could then be used in PCR to amplify family 9 glycosyl hydrolase genes from Tok7B.1. Two primers were designed, the first, tokcela, bound to DNA coding for the peptide sequence QKAIMFYEF, and tokcelr, which bound in the reverse orientation (with respect to the gene sequence) to DNA coding for the peptide sequence DYNAGFVGAL (Figure 3).

[0091] The tokcela and tokcelr primers were used to amplify an approximately 1300bp PCR product from Tok7B.1. This product was ligated into M13 mp10 (Messing, 1983), transformed in *E coli* strain JM101 and plated to give individual recombinant plaques. In order to test whether the PCR product was generated from a single gene, or from multiple genes, PCR product was reamplified from individual plaques using the M13 forward and reverse primers then mapped by restriction digestion with *Tsp*509I. A total of 12 individual PCR products were restriction mapped and all showed identical restriction patterns. Six of these PCR products were sequenced and all showed identical DNA sequence. This data indicated that all cloned PCR products were amplified from a single family 9 glycosyl hydrolase gene present on the genome of Tok7B.1. In order to obtain the complete *celE* gene sequence, new PCR primers were designed to allow genomic walking upstream and downstream of the region covered by the 1300bp PCR product (Figure 4A). Standard subcloning and DNA sequencing techniques were used to obtain 6416bp of DNA sequence containing the entire *celE* gene sequence plus flanking upstream and downstream sequence (Figure 4B). The complete DNA sequence and translated peptide sequence of the *celE* gene is given in Sequence #2.

Genomic Library construction and screening

[0092] Genomic DNA from Tok7B.1 was partially digested with the restriction endonuclease *Tsp*509I to give DNA fragments in the size range of 6-8kb. These fragments were then ligated into *Xho*I-digested λZapII (Stratagene, 11011 North Torrey Pines Road, La Jolla, CA 92037, USA) then packaged and plated according to protocols supplied by Stratagene. Individual plaque isolates shown to contain genomic inserts using the blue/white lacZ complementation system present in λZapII were replated, and a total of 1600 genomic insert containing plaques were screened for thermophilic cellulase and xylanase activity at 70°C using the substrate overlay method of Teather and Wood (1982). Cellulase activity was detected using the soluble cellulose derivative carboxymethyl cellulose (CMC). Plaques were also screened for cellolobiohydrolase activity using the chromogenic substrate methylumbelliferyl cellobioside (MUC) as described by Saul et al. (1990).

[0093] Two positive λZapII plaques, designated W2-4 and N17, were isolated which expressed thermophilic xylanase and/or cellulase activity (Figure 5A). These recombinant phage were converted to Bluescript SK- plasmids using the standard Exassist excision procedure described by Stratagene. Each plasmid was restriction mapped using a range of restriction endonucleases. Common restriction endonuclease digestion patterns indicated that W2-4 and N17 contained common overlapping DNA from the same region of the Tok7B.1 genome (Figure 5A).

DNA sequencing and sequence analysis of the Tok7B.1 *celB* and *celA* genes

[0094] The recombinant DNA from W2-4 and N17 was partially sequenced by creating simple plasmid deletions using known restriction sites within the plasmid insert (Gibbs, et al. 1991). Initial DNA sequence homology comparison data indicated a gene coding for a multidomain enzyme with a xylanase and a cellulase domain and several internal cellulase binding domains (CBD). The Genomic DNA contained by W2-4 was sequenced in full, and portions of N17, by subcloning and sequencing internal restriction fragments and using synthesized DNA oligonucleotide primers (primers are listed in Table I). Analysis of the complete sequence of W2-4 showed that the DNA contained a complete gene, *celB*, coding for a nine-domain protein designated CelB. The 3'-portion of a further gene was observed to lie upstream of the *celB* gene. This gene, designated *celA*, shared at least 1 domain in common with the *celB* gene. The complete coding sequence of *celA* was obtained using Genomic Walking PCR (GW-PCR) as described by Morris et al. (1994). Representative GW-PCR products spanning the region of the *celA* gene are depicted in Figure 5B. The complete DNA sequence containing the *celA* and *celB* genes is depicted in Figure 5C, with each gene shown according to its translated domain structure. The complete DNA sequence and translated peptide sequence of the *celA* and *celB* genes is given in Sequence # 1. The translated product of the *celB* gene matches perfectly with two amino-terminal sequences obtained for native cellulolytic peptides secreted by Tok7B.1 (Figure 2, peptides B2 and B4), implying that the *celB* gene expresses one of the major cellulases secreted by Tok7B.1.

[0095] A complete summary of the protein domain structures of CelA and CelB is given in Figure 6.

[0096] The complete *celE* gene was observed to code for a large multidomain-multicatalytic enzyme with a putative length of 1751 amino-acids (unprocessed) and is composed of at least 10 discrete functional domains based on homology comparisons (figure 6). The family 9 glycosyl hydrolase domain is the amino-terminal domain of the full length CelE, while the central domains of CelE (domains 4-9, figure 6) are virtually identical to the central domains of CelB

(domains 3-8, figure 6), the only exception being the relative lengths of each PT-linker. The carboxy-terminal domain of CelE (domain 10, figure 6) is homologous to the carboxy-terminal endoglucanase domain (family 44 glycosyl hydrolase) of ManA from *C. saccharolyticus*. This domain can degrade xylan as well as carboxymethylcellulose (Gibbs et al. 1991) and activity assays have shown that the carboxy-terminal domain of Tok7B.1 CelE is also an endoglucanase with weak xylanase activity.

Identification of further Tok7B.1 cellulase genes using GW-PCR, *celC* and *celH*

[0097] In the process of obtaining the complete coding sequence of the Tok7B.1 *celE* gene further ORFs were identified upstream of this gene. Homology comparisons indicated that these genes also coded for cellulolytic enzymes. GW-PCR was used to obtain DNA sequence from upstream of the *celE* (figure 7A.) Two further genes were identified in this way. Both of these genes, designated *celC* and *celH,* code for multidomain, multicatalytic proteins, with the same general structure as CelA, CelB and CelE. As the DNA sequence obtained was not contiguous, long-template PCR (Expand Long template PCR System, Boehringer Mannheim, Australia Pty. Ltd.) was used to amplify DNA between the sequenced regions to confirm that they were contiguous (figure 7A). Approximately 13500bp of genomic DNA upstream of the *celE* gene was partially sequenced.

Identification of *celF* and *celG*

[0098] During the isolation of the complete *celA* gene sequence the primer N17a was used as a genomic walking primer. A number of PCR products were obtained which did not match DNA sequence already obtained for the *celB* and *celA* genes. It was clear from these results that the N17a primer was CelB. Upstream of this second xylanase domain a further gene was identified coding for an enzyme with a carboxy-terminal family 48 glycosyl hydrolase domain. These genes were designated *celF* and *celG* respectively (figure 7B). Oligonucleotide primers specific to the carboxy-terminal end of the *celG* gene and the amino-terminal end of the *celF* gene were synthesized and used in combination with oligonucleotide PCRs which bound to DNA coding for the CBDs found in *celA, celB, celE, celC and celH.* The amplification of PCR products indicated that *celG* and *celF* coded for the proteins with the same basic domain structure of the other Tok7B.1 cellulolytic genes. The amino-terminal domain of *celG* was not identified, the carboxy-terminal of *celF* was identified as a family 48 glycosyl hydrolase with high homology to the carboxy-terminal domains of *celG* and *celC*.

Transfer of Tok7B.1 genes into controlled-expression plasmid vectors

[0099] To facilitate the transfer of Tok7B.1 cellulase genes into controlled-expression plasmid vector the general method of Gibbs et al. (1991) was used. PCR was used to amplify full length cellulase genes (and portions of cellulase genes). Oligonucleotide primers corresponding to each end of the gene were engineered to contain restriction sites allowing directional ligation of restriction digested PCR product into plasmid multiple cloning sites. Table II. lists the oligonucleotides designed for PCR amplification and directional ligation of the various Tok7B.1 genes into controlled expression vectors. Each primer contains one or more restriction endonuclease site(s) to facilitate ligation of PCR product into plasmid vector predigested with the same restriction enzyme, resulting in an in-frame gene fusion between each thermophilic gene and a signal peptide sequence encoded on the vector. The various genes and gene fragments transfer into pJLA602 by this method are shown in Figure 8.

Phylogenetic analysis of Tok7B.1

[0100] The 16S SSU rRNA gene was isolated using PCR. A PCR product was generated using oligonucleotide primers designed to amplify the 16S SSU rRNA gene from all known prokaryotic species. An approximately 1800bp PCR fragment was obtained which was cloned into M13 mpIO in the forward and reverse orientation, and sequenced (Seq #3). The SSU rRNA gene sequence obtained was compared to all genes in the GenBank database. Close homologs of the Tok7B.1 SSU rRNA gene were aligned using the GCG multiple alignment software 'Pileup'. Resulting aligned sequence files were subsequently analyzed using parsimony methods (Swofford, 1993). Figure 9 shows the phylogenetic position of Tok7B.1 amongst cluster D of thermophilic Clostridia (Rainey et al., 1993).

Cloning of individual genes into an *E. coli* expression vector

[0101] From the *celE* and *celB* genes a number of new truncated genes containing either individual cellulase catalytic domains Cel EI or catalytic domains connected to cellulose binding domains by linker sequences, Cel E1/2, CelE1/2/3 and CelB4/5 have been constructed (Table III). Each of the genes have been individually expressed in E. coli using

the bacteriophage T-7 RNA polymerase/promoter system (Studier and Moffatt, 1986).

Expression Cloning of th CelE Domains D2

[0102] The N-terminal CelE endoglucanase catalytic domain (Figure 6) and the first cellulose-binding domain (CBD) (Figure 6) were used to construct expression plasmids pcelE! and pcelEl/2 respectively. These *celE* gene domains were obtained from the M13-mp10 clones, M13celE1 and M13celE1/2. The first step in the cloning process was the PCR amplification of domain 2 or domains 2 plus 3 of the *celE* gene from Tok7B.1 genomic DNA (Figure 10). Unique restriction endoglucanase sites were introduced by the PCR primers at the 5' and 3' ends of the gene fragments. An *SphI* site was incorporated at the 5' end of the native gene at the predicted translational start site, which encodes the translational ATG start codon, and *BglII* sites were incorporated at the 3' ends of the specific gene domains at convenient locations. Translational stop codons were introduced just upstream of the *BglII* sites. The PCR fragments were blunt end ligated directly into *SmaI* digested M13mp10 vector, (Messing, 1983) to give the clones M13-celE1 and M13-celE1/2 (Figure 11).

[0103] Using the pET9a vector (Novagen) *E. coli* expression plasmids were constructed. The plasmid utilizes the T7 Polymerase promoter for gene expression, (Studier, et al., 1990). An intermediate construct was employed to facilitate the cloning process. The *celE1/2/3* gene was amplified using PCR, the forward direction primer tokcbdf, and the reverse direction primer tokcel (Figure 11). The forward primer, tokcbdf introduces a *NdeI* site at the 5' end of the mature *celE* gene and thereby encodes the translational ATG start codon. The introduction of the *NdeI* restriction site changed the first two amino acids encoded in the mature sequence from GT to AA Table III. The reverse PCR primer, tokcel, was homologous to the native gene sequence at the *NdeI* site in CBD domain 3. The PCR fragment was digested with NdeI and gel-purified with silica gel technology using a Qiaex II gel extraction kit from Qiagen Inc. The fragment was ligated into the *NdeI* site of the pET9a vector (Figure 12). The resulting plasmid, pMcelE-NdeI, was digested with *PstI* and *BamHI* and the vector fragment was isolated from the digest by agarose gel electrophoresis and silica gel purification. The M13-celE1 and M13-celE1/2 clones were digested with *PstI* and *BglII* and the resulting *celE* gene fragments, *celE1* and *celE1/2*, were isolated from the digest by agarose gel electrophoresis and silica gel purified (Figure 12). The fragments were ligated to the *PstI-BamHI* digested pcelE-*NdeI* plasmid to form the final clones, pMcelEl and pMcelE1/2 (Figure 12). Both the *BglII* and *BamHI* restriction enzymes produce compatible sticky ends but these sites are lost upon ligation.

Expression Cloning of the CelE D2/3/4/5

[0104] The pcelE1/2/3 plasmid encodes the first catalytic domain of the celE gene plus the first two cellulose-binding domains D3 and D5 (Table III) in a pET9a expression vector. The catalytic domain D2 and CBD D3 used in the construction of the pcelE1/2/3 expression plasmid was obtained from the pcelEl/2 plasmid. The second cellulose-binding domain D5 was obtained from the pRR9 plasmid (Figure 8). The construction of the final plasmid required a three-way ligation that is outlined in Figure 13.

[0105] The entire native *celE* gene was amplified by PCR from genomic Tok7B.1 DNA using the tocelef forward primer and the tokceler3 reverse primer Table II. The PCR primers contained an *SphI* site in the forward primer, which introduces the ATG translational start codon, and a *SalI* site in the reverse primer. The PCR fragment was digested with *SphI* and *SalI* and cloned into the *SphI* and *SalI* sites of the polylinker of the *E. coli* expression vector pJLA602, to produce the pRR9 plasmid (Figure 8). To obtain the gene fragment encoding domains 4 and 5 for ligation with the pcelE1/2 plasmid, the region from the *NcoI* site in D3 through D5 was PCR amplified from the pRR9 plasmid (Figure 13). Tokcelef, the forward primer, was homologous to the *celE* sequence at the *NcoI* site and the tokcelebamr reverse primer was homologous to the end of D5, the second CBD in *celE* and introduced a *BamHI* cloning site. This PCR fragment was digested with *NcoI* and *BamHI* and purified. The *celE* fragment from D2 to the 5' end of D3 at the *NcoI* site was isolated from the plasmid pcelEl/2 (Figure 13). The plasmid was digested with NdeI and *NcoI* and the *celE* fragment was isolated from the vector fragment by gel electrophoresis and silica gel technology. The vector, pET9a, was digested with *NdeI* and *BamHI* and purified by gel electrophoresis and silica gel technology. The two *celE* fragments were ligated to the pET9a expression vector in a three-part ligation to produce the pcelEl/2/3 plasmid (Figure 13).

Expression cloning of CelB4/5

[0106] A plasmid that expressed the CelB4/5 protein of the Tok7B.1 *celB* gene was constructed in the *E. coli* expression vector, pET9a, as described below. Domains 7, 8 and 9 containing a CBD and catalytic domain were PCR amplified from the Tok7B.1 genomic DNA using primers tokcbdf and tokcelbr. These primers incorporated into the PCR fragment a unique 5' *NdeI* site by the forward primer and a unique 3' *BamHI* site (Figure 8). The fragment was digested with *NdeI* and *BamHI* and ligated into the NdeI and *BamHI* digested pJLA602 expression vector to produce the pRR6

plasmid (Figure 14). The pRR6 plasmid was digested with *NdeI* and *BamHI* and the *celB* gene was purified from the vector fragment by gel electrophoresis and silica gel technology. The pET9a vector was digested with *NdeI* and *BamHI* and purified by gel electrophoresis and silica gel technology. The two fragments were ligated together to produce the pcelB4/5 plasmid (Figure 14).

Expression Cloning of CelB3/4/5

[0107] The CBDs of the celE gene, domains 4 & 5, (Figure 8) are very homologous to the CBDs of the celB gene, domains 3 & 4, (Figure 8). Also, the two CBDs within the genes are very homologous to each other. This homology is useful for the construction of the pcelB3/4/5 construct in the E. Coli expression vector pET9a. A homologous region of domain 3 of the celB gene is cloned from the celE gene construct. This is done by taking advantage of a BglII site in each of the homologous celE CBD domains 4 & 5. This BglII fragment is isolated by restriction digest from the celE construct pRR10 which encodes domains 3,4,5, & 6 of the celE gene, Figure 8, in the pJLA602 expression vector. This BglII fragment contains the 3' portion of celE Domain 3 and the 5' portion of celE Domain 4. This BglII fragment is ligated into the BglII site of Domain 4, the CBD, of pcelB4/5. The resulting plasmid is pcelB3/4/5.

Expression cloning of CelE3/B5

[0108] This clone is constructed in the E. Coli expression vector pET9a. Domain 3 of the celE gene is PCR amplified from pcelE1/2/3. The forward and reverse primers incorporated into the PCR fragment provide unique 5' NdeI and 3' BstEII sites. The PCR fragment is digested with NdeI and BstEII and ligated to the pcelB4/5 vector which is digested with NdeI and BstEII and gel purified (Figure 14A). The NdeI and BstEII digest of the pcelB4/5 results in the removal of the native celB CBD as well as 29 amino acids from the PT linker.

Fermentation of the *E. coli* expressing cloned cellulase genes

[0109] The pcel E1, pcel E1/2, pcel E1/2/3, pcelB4/5, pcelB3/4/5 and pcel E3/B5 expression plasmids were transformed into *E. coli* DE3-BL21 (Stratagene Corp.). Transformants were grown at 37° C to an OD600 of 1.0 in 250 mL of L-broth containing 50 µg/ml Kanamycin. The 250 ml of L-broth was then used to inoculate a 20 L Chemap fermentor containing 12 liters of media. The fermentation media consisted of 12 g/L of tryptone, 24 g/L yeast extract, KH_2PO_4 2.3 g/L, 12.5 g/L K_2HPO_4, 1 mL/L Antifoam 289 (Sigma), 4g/L glycerol, 1 mL/L 1.0 M $MgSO_4$.$7H_2O$ and 50 µg/mL Kanamycin. The transformants were grown at 37° C to an OD600 of approximately 12 and then expression was induced by the addition of IPTG at a concentration of 95 mg/L. After a 3h induction the cells were harvested by centrifugation in 500ml bottles at 7,000 x g for 10 min. A typical yield from a 12-L fermentation was 300 g of wet cell paste. Cell pellets were then frozen at -80° C prior to lysis and purification of the recombinant proteins.

Purification of the Cel E1 and Cel E1/2 Cellulases

[0110] The *E. coli* fermentation cell pellets were thawed by resuspending the frozen cells in two volumes of 20 mM Tris buffer pH 8.0. The cells were homogenized with a Virtis Virtishear 1200 for 20 min., then lysed by one passage through a Microfluidizer (Microfluidics Corp.) at a pressure of 9600 psi. The lysate was centrifuged at 43,000 x g for 30 min. The pellet was discarded and the supernatant was combined with sufficient ammonium sulfate to make a 1 molar solution. The ammonium sulfate solution was stored overnight at 4° C then centrifuged at 15,000 x g for 20 minutes. The supernatant was then chromatographed on phenyl sepharose. The column (5 x 10 cm) was washed with 10 mM Tris pH 8.0, 1.0 M ammonium sulfate. After the column effluent had an A280 of less than 0.1 AU, the protein was eluted with a 300 mL linear gradient from 1.0 M to 0 M ammonium sulfate. This column eluent was used in the application testing. Each of the constructs tested in the application was electrophoresed on a 12% polyacrylamide gel and then blotted to an Immobilon membrane and N-terminally sequenced. Figure 16 shows the expected N-terminal sequenced versus the sequence found upon Edman degradation.

Purification of the CelB5 and CelB4/5 Cellulases

[0111] When the Cel B4/5 protein purification described below is carried out in the presence of a protease inhibitor cocktail consisting of phenymethyl sulfonyl fluoride, EDTA and Aprotinin, the full length protein, CelB4/5, consisting of the CBD, PT linker region and catalytic domain is purified. However, in the absence of the protease cocktail, the linker region is cleaved to yield the Cel B5 endoglucanase domain alone, without the CBD or PT linker domains.

[0112] For purification of the CelB4/5, 280 g of cells expressing celB4/5 were thawed in three volumes of 10 mM Tris, pH 7.0 in the presence of the protease cocktail described above. The thawed cells were virtisheared for 20 min.

then lysed as before by a single pass on the Microfluidizer. The lysate was centrifuged for 10 min. at 3,500 x g. The resulting supernatant (820ml) was heated in a 50° C water bath for 10 minutes, then centrifuged for 20 minutes at 3,000 x g. Sufficient $(NH_4)_2SO_4$ was added to give a 20% saturated solution, the solution was centrifuged for 30 min. at 3,000 x g and the pellet discarded. More $(NH_4)_2SO_4$ was added to the supernatant until the solution was 35% saturated, the solution was centrifuged for 30 min. at 3,000 x g and the supernatant discarded. The pellet was resuspended in 10 mM Tris pH 8.0, 0.5 mM EDTA, 1 mM Aprotinin.

[0113] The solution was chromatographed on a 430 ml DEAE column (5 cm x 20 cm) and eluted with a two-step NaCl gradient. Step one of the elution profile was 0 to 150 mM NaCl wash in 300 ml, step two was a wash of 150 mM to 260 mM NaCl linear gradient in 1200 ml. The CMCase activity eluted between 750-950 ml and gave 1.5 g of CelB4/5 protein.

[0114] CelB5 was purified in an identical manner except the only protease inhibitor added to the cell lysate supernatant was 1mM PMSF. CelB5 eluted in an identical manner from the DEAE column. The total protein purified was 1g from about 280 g of cells.

Purification of CelB3/4/5

[0115] 400g of frozen cells are thawed in 800 ml of 10 mM Tris, pH 8.0, 0.5 mM EDTA. The cells are lysed by one pass through the Microfluidizer at 12,000 psi. The lysed sample is then centrifuged at 7,800 x g for 50 min. To the supernatant (950 ml) is added slowly 100.7 g of $((NH_4)_2SO_4$ to give a 20% saturated solution. The solution is stirred overnight for 12h at 4° C . The precipitated proteins were removed by centrifugation for 30 minutes at 14,000 x g. The remaining supernatant is brought up to 40% $(NH_4)_2SO_4$ and left to stir for 48 h at 4° C. The precipitate is pelleted by centrifugation for 30 min at 15,000 x g. The pellet is resuspended in 20 mM Mes pH 6.0 . The conductivity is reduced to less than 3 ohms/cm^2 by diafiltration using a 30kD Filtron membrane. The dialysate is centrifuged to remove any precipitate and chromatographed on S-sepharose (10 cm x 6 cm) and eluted with a linear salt gradient from 0.1M to 0.35M. Fractions containing activity of greater than 200 units/mL are pooled. The final pool contains 720 mg of protein which is approximately 52 % pure as determined by densitometry scanning of a Coomassie stained 12% SDS PAGE of the pool.

Purification of CelE3/B5

[0116] 400 gm of E. coli DE3-B121 are thawed in 10 mM Tris, pH 8.0, 0.5 mM EDTA. The cells are lysed by passage through the microfluidizer at 12,000 psi. The precipitate is removed by centrifugation of the lysate for 30 min at 8,000 x g. To the supernatant is then added solid $(NH_4)_2SO_4$ to give a 20 % saturated solution. The precipitate is removed by centrifugation at 14,000 x g for 30 min and the supernatant was loaded on a phenyl sepharose column 6 cm x 10cm. The protein is eluted with a 2L reverse linear gradient from 1 M to 0 M $(NH_4)_2SO_4$ in lysis buffer. The bulk of the activity is collected in three fractions. Each of the fraction contains 250 ml. Each of the fraction is analyzed for the activity.

[0117] The conductivity is reduced to less than 3 ohms/cm^2 by diafiltration using a 30kD Filtron membrane with a 10 mM Immidazole pH 7.0. The dialysate is chromatographed on S-sepharose (10 cm x 6 cm) and eluted with a linear salt gradient from 0 M to 0.23 M. Fractions containing activity of greater than 250 units/mL are pooled. The final pool contains 720 mg of protein which is approximately 86.9 % pure as determined by densitometry scanning of a Coomassie stained 12% SDS PAGE of the pool.

pH rate profiles of purified Cellulases

[0118] The pH rate profiles and thermostability of the cellulases were determined. These data serve to define the pH extremes at which an enzyme could be used in an application. Cellulases were assayed at 50° C for the determination of the pH rate profiles. The catalyzed rates of reaction at each pH are expressed as fractions of the fastest observed rate. This is calculated by dividing the rate of reaction at each pH by the highest reaction rate observed at any pH, the highest reaction rate is therefore plotted as 1.0. The CMC substrate and buffer in each case was made with an appropriate buffer for each pH being tested. The following buffers were employed for each of the assays, at pH 3.0 sodium tartrate (25 mM), pH 4.0 sodium tartrate (50 mM), pH 5.0 sodium acetate (50 mM), pH 7.0 sodium phosphate (50 mM), pH 9.0 glycine (50 mM), pH 10.0 glycine (50 mM), pH 11.0 CAPS (50 mM), pH 12.0 sodium phosphate (50 mM). 2% CMC was made up at each pH in the buffers listed. No more than 10 µl of enzyme was added to the total reaction mixture of 0.5 ml so that the pH of the reaction would not be effected.

Thermal Stability of Cellulases

[0119] The thermal stability of these proteins is summarized in Table IV. The addition of CBDs to the catalytic domains

has different effects on the thermal stability of the protein constructs. The CelE1 was dramatically stabilized by the addition of the cellulose binding domains, there is a 25° C increase in the stability of the CelE1/2 relative to CelE1.

[0120] Assays to determine the thermostability of the cellulases with time were carried out in one of two ways depending on the temperature at which the studies were done and the time of incubation. At temperatures of up to 80° C or if the samples were incubated for less than two minutes then stability studies were done by protocol 1. An aliquot (40 µl) of the purified cellulase was diluted into an aliquot (200 µL) of incubation buffer, 50 mM sodium phosphate buffer at pH 7.0, that was preheated in an 80° C water bath. At the specified time points aliquots (25 µl) were withdrawn from the diluted sample incubated at the designated temperature and diluted into 475 µl of ice cold incubation buffer. Each of the time points was then assayed to determine the remaining cellulase activity using the standard CMCase assay.

[0121] Protocol 2 was used when incubations of above 80° C were done for a time in which any assay point exceeded two minutes of incubation time. In this case sufficient cellulase for an individual CMCase assay was placed in a tube and preheated to 80° C. At time 0 the samples were then transferred to a water bath at a higher temperature for example 85° or 90°. At the designated time points the samples were withdrawn and placed in an ice water bath. Each of the time end points was then assayed to determine the remaining cellulase activity with time using the standard CMCase assay.

Structural characterization of purified cellulases

[0122] Characterization of the CelB5 protein by MALDI-TOF and N-terminal sequencing shows the linker domain is clipped between T999 and A1000 in the full length CelB protein sequence and that the two C-terminal amino acids K1424 and N1425 are also proteolyzed (Figure 15). The N-terminal sequence of the expressed proteins were determined using the techniques of Matsudaria (1987) in which proteins were electrophoresed on SDS PAGE, blotted to PVDF membranes and then N-terminally sequenced by Edman degradation (Figure 15).

Application Testing of Tok7B.1 Cellulase Constructs

[0123] The purified enzymes were tested in the denim stone-wash application, under the same conditions that were used in the initial evaluation of the cellulase supernatants. Results are shown in Table IV. Cellulase constructs that gave a stonewashing effect and showed a dose dependent increase in abrasion with increasing concentrations of enzyme were lacking a cellulose binding domain. Results demonstrated the CelB5 and CelE1 protein constructs gave the best stonewash effect.

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Annex to the description

SEQUENCE LISTING

(1) GENERAL INFORMATION

(i) APPLICANT
(A) NAME: CLARIANT FINANCE (BVI) LIMITED
(B) STREET: Citco Building, Wickhams Cay, P.O. Box 662
(C) CITY: Road Town
(D) STATE OR PROVINCE: Tortola
(E) COUNTRY: British Virgin Islands
(F) POSTAL CODE:

(ii) TITLE OF THE INVENTION: Truncated Cellulase Compositions

(iii) NUMBER OF SEQUENCES: 47

(iv) COMPUTER-READABLE FORM:
(A) MEDIUM TYPE: Diskette
(B) COMPUTER: IBM Compatible
(C) OPERATING SYSTEM: DOS
(D) SOFTWARE: FastSEQ for Windows Version 2.0

(v) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: 98810919.5

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11707 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6416 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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TTGCAGCCTG ACGTGAATGA TAACTATGTA TATATGGATG AGTTTATTAA CTATCTGATT   4800
AATAAGTATG GTCGATCATC GTCTGCAACG GGAATTAAAG GATATATACT TGACAACGAG   4860
CCGGACTTAT GGTTTACTAC TCATCCGCGA ATTCATCCAC AGAAGGTAAC CTGCAGTGAA   4920
TTGATAAATA AATCGGTGGA GCTGGCGAAA GTAATAAAGA CACTTGATCC AGATGCAGAA   4980
ATTTTTGGAC CTGCATCGTA TGGTTTTGTG GGATATTTAA CATTGCAGGA TGCACCTGAC   5040
TGGAATCAGG TTAAAGGAAA TCACAGATGG TTTTTGAGCT GGTACCTTGA GCAGATGAAG   5100
AAAGCATCGG ATAGTTTTGG GAARAGGTTA TTGGATGTAC TTGACATACA CTGGTACCCG   5160
GAGGCGCAGG TTGGCGGTGT GCGAATATGC TTTGACGGTG AAAATAGTAC TTCAAGGGAT   5220
GTGGCAATAG CGAGGATGCA GGCACCGAGA ACGCTATGGG ATCCGACATA TAAAACCACC   5280
CAGAAAGGTC AGATAACAGC GGGAGAAAAT AGCTGGATAA ACCAATGGTT TCCAGAGTAT   5340
CTTCCACTGC TTCCCAATAT AAAGGCAGAT ATAGACAAGT ATTATCCTGG TACCAAACTT   5400
GCTATAACTG AGTTTGATTA TGGAGGGAAG GACCATATAT CGGGAGGAAT AGCTTTAGCA   5460
GATGTGTTAG GGATATTCGG CAAGTATGGA GTATACATGG CAGCAAGATG GGGAGATTCG   5520
GGGAGCTATG CACAGGCGGC GTACAACATT TATCTCAACT ATGATGGGAA AGGTTCGAGA   5580
TACGGTTCAA CGTGTGTGAG CGCTGAGACA ACTGACGTTG AGAACATGCC GGTATATGCT   5640
TCAATTGAGG GAGAAGATGA TTCGACTGTG CATATTATAT TAATTAACAG GAATTATGAC   5700
AGGAAACTGA AGGCAGAGAT AAAGATGAAT AATACCAGGG TATACACAGG TGGAGAGATA   5760
TACGGATTTG ACAGTACAAG CTCTCAGATC AGGAAGATGG GAGTGCTCAG TAATATACAA   5820
AACAACACAA TCACCATAGA AGTTCCAAAT CTGACGGTAT ACCATATTGT TTTAACTTCT   5880
TCAAAGTAGA TTAAAGAATA AAAATGGAGA CACTGCTGCA TGGTAAAAGT TGAGATGTGC   5940
AGCAGTGTCT CATAATCACT AATCTAATAC AGTTAGAGAT GTTAAATTAT AAAACAGACG   6000
ATAACTTTGT TTTAAATGAT TGNNAGTCGG ANTTCTNNTG ATTAAAACAT NAGAAANTTG   6060
TNATANTNGA CTTTAATTNT NGCNNATAAA CGTAAATGGA TTCAATNACN WTACRATTTN   6120
CRTAATCTAW AAGRAGCACA GAGAAATATT ACATAGGAGG ATGTATCAAT AAATGATAGA   6180
TAAAAAGATA ATTGCTGTTA CAATTTTART AATGGTAACA TACTTTTTAG TACAAATATC   6240
RACTATAGGT GCACGGAATA TACCAGAGAC ATANTGGATA CCGCTGGATA TAGATACAAT   6300
AAGTATTGAC CTGGGCWAGN AGCCATATGT GANAGAATTT ATAGTATATT TTGGATATGG   6360
CGGAGGCAAA ATAGASTGTC WGTTTTATAG AGACAATACT TTGGCATTMT ACATCA       6416
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
CCTTTATGAA TTCATTTACT GACTGCTA                                      28
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CTTCCCTCGA GAATTCACAC ACCCACTTTT G 31

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TACCCCTCGA GAATTCCTAT TTACTCATTA 30

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CTACACCCAT GGTAACCCCC GATGTTAA 28

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

AAATGCTCGA GTAAAAGTGA ACAAGCA 27

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

ATGTGTCCAT GGCATTAATT ATTTTTGTTG 30

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

ATGCAAGGCA TGCAAGCAAT TAAGAGGGTT G 31

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

TCAACAAAGA TCTAATCATT TGTGGGTGTT TC 32

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GTGCAGCTCG AGCTCCTCCC GGCTCCTGCC CCCA 34

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GAGGAACGGT CATATCAAGG TATGGTATGC GAATGGGAA 39

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GAGGAGGAGC ATGCAGATCA AGGTATGGTA TGCGAATG 38

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

TTTAGCATGC TGAGGAAATA CAAAG 25

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

AGTTAGTGGC ATGCAAAAGA GAGTTTTAAG G 31

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GAAGTATGGA TCCATTTATT AATTCTTTGG G 31

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

TACAATTTTA GCCATGGTAA CATACTTTTT AG 32

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

GCAGCAGTGT CGACATTTTT ATTCTTTAAT CTAC 34

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GTGGATGAGA TCTAACCCGG CTCTAAACCC CA 32

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

TTGAACTTCC CCATGGCAGA ATTTTTACAA ATTGG 35

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

TGTATCCCAT GCCGTCTT 18

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

CAAAAAGCAA TTATGTTTTA TGAATT 26

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

TGGTGCTGGC AATGTTGAGT TGGC 24

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

TCGGTAGTGC CACTTTCAAA TCCA 24

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

CAAAGCAGAC GAATCTGTGC GTGGTATGCA ATATAC 36

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

AGCTGAGCAG CGGAGTGA 18

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

TCCACTCACT CCGCTGCT 18

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

GTTCTGATAC TGTCCAAG 18

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

ACAGGCGGCG TACAACAT 18

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

TTGAGGGATA TGGTGACC 18

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

GAGAAACATA TCCTGCAA 18

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

CCCATTTTAT ACCCAGGC 18

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

TCTTGAGCAG CCATTGGA 18

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

GATGGCCAGT TCACGTTTAT ATGG 24

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

AGCACTGGTT GGTGGTCCTG GTAG 24

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

GATTGACGGG TTACAATTGG GAGAAC 26

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

AGWGCACCNA CAAATCCGGC ATTGTARTC 29

(2) INFORMATION FOR SEQ ID NO:38:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

CTCCAGAATG TCATTTGTAA GATACAT 27

(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

GGGAATTCCA TATGGCGGCG TATAATTACG GTGAG 35

(2) INFORMATION FOR SEQ ID NO:40:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

TATTATTATC ATATGCGGC 19

(2) INFORMATION FOR SEQ ID NO:41:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

CCAGAGTATC ACAGACAC 18

(2) INFORMATION FOR SEQ ID NO:42:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

CCTGGATCCC TACGCTCCTC CCGGCTC 27

(2) INFORMATION FOR SEQ ID NO:43:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1426 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

```
Met Lys Lys Arg Val Leu Arg Phe Val Ser Arg Leu Ile Leu Ala Val
 1               5                  10                  15
Phe Ile Met Ser Ile Ser Leu Val Gly Ser Met Ser Tyr Phe Pro Val
            20                  25                  30
Lys Thr Glu Ala Ala Pro Asp Trp Ser Ile Pro Ser Leu Trp Glu Ser
        35                  40                  45
Tyr Lys Asn Asp Phe Lys Ile Gly Val Ala Ile Pro Ala Arg Cys Leu
    50                  55                  60
Ser Asn Asp Thr Asp Lys Gln Met Val Leu Lys His Phe Asn Ser Ile
65                  70                  75                  80
Thr Ala Glu Asn Glu Met Lys Pro Glu Ser Leu Leu Ala Gly Gln Thr
                85                  90                  95
Ser Thr Gly Leu Ser Tyr Arg Phe Ser Thr Ala Asp Thr Phe Val Asn
            100                 105                 110
Phe Ala Asn Thr Asn Asn Ile Gly Ile Arg Gly His Thr Leu Val Trp
        115                 120                 125
His Asn Gln Thr Pro Asp Trp Phe Phe Arg Asp Ser Ser Gly Gln Met
    130                 135                 140
Leu Ser Lys Asp Ala Leu Leu Ala Arg Leu Lys Gln Tyr Ile Tyr Asp
145                 150                 155                 160
Val Val Gly Arg Tyr Lys Gly Lys Val Tyr Ala Trp Asp Val Val Asn
                165                 170                 175
Glu Ala Ile Asp Glu Ser Gln Pro Asp Gly Tyr Arg Arg Ser Thr Trp
            180                 185                 190
Tyr Gln Ile Cys Gly Pro Glu Tyr Ile Glu Lys Ala Phe Ile Trp Ala
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                195                 200                 205
His Glu Ala Asp Pro Asn Ala Lys Leu Phe Tyr Asn Asp Tyr Asn Thr
    210                 215                 220
Glu Ile Ser Thr Lys Arg Asp Phe Ile Tyr Asn Met Val Lys Asn Leu
225                 230                 235                 240
Lys Ser Lys Gly Val Pro Ile His Gly Ile Gly Met Gln Ser His Ile
                245                 250                 255
Asn Val Asn Trp Pro Ser Val Ser Glu Ile Glu Asn Ser Ile Lys Leu
            260                 265                 270
Phe Ser Ser Ile Pro Gly Ile Glu Ile His Ile Thr Glu Leu Asp Met
        275                 280                 285
Ser Leu Tyr Asn Tyr Gly Ser Asn Glu Asn Tyr Ser Thr Pro Pro Gln
    290                 295                 300
Asp Leu Leu Gln Arg Gln Ala Gln Lys Tyr Lys Asp Ile Phe Thr Met
305                 310                 315                 320
Leu Arg Lys Tyr Lys Gly Ile Val Thr Cys Val Thr Phe Trp Gly Leu
                325                 330                 335
Lys Asp Asp Tyr Ser Trp Leu Asn Ser Ser Ser Lys Arg Asp Trp Pro
            340                 345                 350
Leu Leu Phe Phe Asp Asp Tyr Ser Ala Lys Pro Ala Tyr Trp Ser Val
        355                 360                 365
Ile Glu Ala Ala Gly Ala Ser Ala Ser Pro Ser Pro Thr Val Thr Ala
    370                 375                 380
Thr Pro Thr Pro Thr Pro Thr Pro Thr Val Thr Val Thr Ala Thr Pro
385                 390                 395                 400
Thr Pro Thr Pro Thr Gly Thr Pro Gly Thr Gly Ser Gly Leu Lys Val
                405                 410                 415
Leu Tyr Lys Asn Asn Glu Thr Ser Ala Ser Thr Gly Ser Ile Arg Pro
            420                 425                 430
Trp Phe Lys Ile Val Asn Gly Gly Ser Ser Ser Val Asp Leu Ser Arg
        435                 440                 445
Val Lys Ile Arg Tyr Trp Tyr Thr Val Asp Gly Asp Lys Pro Gln Ser
    450                 455                 460
Ala Val Cys Asp Trp Ala Gln Ile Gly Ala Ser Asn Val Thr Phe Asn
465                 470                 475                 480
Phe Val Lys Leu Ser Ser Gly Val Ser Gly Ala Asp Tyr Tyr Leu Glu
                485                 490                 495
Val Gly Phe Ser Ser Gly Ala Gly Gln Leu Gln Pro Gly Lys Asp Ala
            500                 505                 510
Gly Asp Ile Gln Val Arg Phe Asn Lys Asn Asp Trp Ser Asn Tyr Asn
        515                 520                 525
Gln Ala Asp Asp Trp Ser Trp Leu Gln Ser Met Thr Asp Tyr Gly Glu
    530                 535                 540
Asn Ala Lys Val Thr Leu Tyr Val Asp Gly Val Leu Val Trp Gly Gln
545                 550                 555                 560
Glu Pro Gly Gly Ala Thr Pro Ala Pro Thr Ala Thr Ala Thr Pro Thr
                565                 570                 575
Pro Ile Pro Thr Ala Thr Val Thr Pro Thr Pro Thr Ala Thr Pro Thr
            580                 585                 590
Ser Thr Pro Arg Pro Thr Ala Thr Ala Thr Pro Thr Pro Thr Val Ser
        595                 600                 605
Ala Thr Pro Thr Pro Ala Pro Thr Ala Ser Pro Val Gly Gly Ser Tyr
    610                 615                 620
Trp Thr Pro Ser Glu Ser Tyr Gly Ala Leu Lys Val Trp Tyr Ala Asn
625                 630                 635                 640
Gly Asn Leu Ser Ser Pro Thr Asn Val Leu Asn Pro Lys Ile Lys Ile
                645                 650                 655
Glu Asn Val Gly Thr Thr Ala Val Asp Leu Ser Arg Val Lys Val Arg
            660                 665                 670
Tyr Trp Tyr Thr Ile Asp Gly Glu Ala Thr Gln Ser Val Ser Val Ala
        675                 680                 685
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Ser Ser Ile Asn Pro Ala Tyr Ile Asp Val Lys Leu Gly Ala Asn Ala
    690                 695                 700
Gly Gly Ala Asp Tyr Tyr Val Glu Ile Gly Phe Lys Ser Gly Ala Gly
705                 710                 715                 720
Val Leu Ala Ala Gly Gln Ser Thr Lys Glu Ile Arg Leu Ser Ile Gln
                725                 730                 735
Lys Gly Ser Gly Ser Tyr Asn Gln Ser Asn Asp Tyr Ser Val Arg Ser
            740                 745                 750
Ala Thr Gly Tyr Ile Glu Asn Glu Lys Val Thr Gly Tyr Ile Asp Asp
        755                 760                 765
Val Leu Val Trp Gly Arg Glu Pro Ser Arg Asn Ala Gln Ile Lys Val
    770                 775                 780
Trp Tyr Ala Asn Gly Asn Leu Ser Ser Pro Thr Asn Val Leu Asn Pro
785                 790                 795                 800
Lys Ile Lys Ile Glu Asn Val Gly Thr Thr Ala Val Asp Leu Ser Arg
                805                 810                 815
Val Lys Val Arg Tyr Trp Tyr Thr Ile Asp Gly Glu Ala Thr Gln Ser
            820                 825                 830
Val Ser Val Thr Ser Ser Ile Asn Pro Ala Tyr Ile Asp Val Lys Phe
        835                 840                 845
Val Lys Leu Gly Ala Asn Ala Gly Gly Ala Asp Tyr Tyr Val Glu Ile
    850                 855                 860
Gly Phe Lys Ser Gly Ala Gly Val Leu Ala Ala Gly Gln Ser Thr Lys
865                 870                 875                 880
Glu Ile Arg Leu Ser Ile Gln Lys Gly Ser Gly Ser Tyr Asn Gln Ser
                885                 890                 895
Asn Asp Tyr Ser Ile Arg Ser Ala Asn Ser Tyr Ile Glu Asn Glu Lys
            900                 905                 910
Val Thr Gly Tyr Ile Asp Gly Ala Ile Val Trp Gly Arg Glu Pro Ser
        915                 920                 925
Arg Gly Thr Lys Pro Ala Gly Val Val Thr Pro Thr Pro Ala Pro Thr
    930                 935                 940
Pro Thr Ser Thr Pro Thr Pro Ile Pro Thr Thr Thr Pro Thr Pro Thr
945                 950                 955                 960
Pro Thr Pro Thr Val Thr Val Thr Pro Thr Ser Thr Pro Thr Pro Val
                965                 970                 975
Ser Ser Ser Thr Pro Thr Pro Thr Ala Thr Pro Thr Pro Thr Pro Ser
            980                 985                 990
Ile Thr Ile Thr Pro Ala Pro Thr Ala Thr Pro Thr Pro Thr Pro Ser
        995                 1000                1005
Val Thr Asp Asp Thr Asn Asp Asp Trp Leu Phe Ala Gln Gly Asn Lys
    1010                1015                1020
Ile Val Asp Lys Asp Gly Lys Pro Val Trp Leu Thr Gly Val Asn Trp
1025                1030                1035                104
Phe Gly Phe Asn Thr Gly Thr Asn Val Phe Asp Gly Val Trp Ser Cys
                1045                1050                1055
Asn Leu Lys Ser Ala Leu Ala Glu Ile Ala Asn Arg Gly Phe Asn Leu
            1060                1065                1070
Leu Arg Val Pro Ile Ser Ala Glu Leu Ile Leu Asn Trp Ser Lys Gly
        1075                1080                1085
Ile Tyr Pro Lys Pro Asn Ile Asn Tyr Tyr Val Asn Pro Glu Leu Glu
    1090                1095                1100
Gly Leu Thr Ser Leu Glu Val Phe Asp Phe Val Val Lys Thr Cys Lys
1105                1110                1115                112
Glu Val Gly Leu Lys Ile Met Leu Asp Ile His Ser Ala Lys Thr Asp
                1125                1130                1135
Ala Met Gly His Ile Tyr Pro Val Trp Tyr Thr Asp Thr Ile Thr Pro
            1140                1145                1150
Glu Asp Tyr Tyr Lys Ala Cys Glu Trp Ile Thr Glu Arg Tyr Lys Asn
        1155                1160                1165
Asp Asp Thr Ile Val Ala Phe Asp Leu Lys Asn Glu Pro His Gly Lys
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    1170                1175                1180
Pro Trp Gln Asp Ser Val Phe Ala Lys Trp Asp Asn Ser Thr Asp Ile
1185                1190                1195                120
Asn Asn Trp Lys Tyr Ala Ala Glu Thr Cys Ala Lys Arg Ile Leu Ala
                1205                1210                1215
Lys Asn Pro Asn Met Leu Ile Val Ile Glu Gly Ile Glu Ala Tyr Pro
            1220                1225                1230
Lys Asp Asp Val Thr Trp Thr Ser Lys Ser Ser Ser Asp Tyr Tyr Ser
        1235                1240                1245
Thr Trp Trp Gly Gly Asn Leu Arg Gly Val Lys Lys Tyr Pro Ile Asn
    1250                1255                1260
Leu Gly Gln Tyr Gln Asn Lys Val Val Tyr Ser Pro His Asp Tyr Gly
1265                1270                1275                128
Pro Leu Val Tyr Gln Gln Pro Trp Phe Tyr Pro Gly Phe Thr Lys Asp
                1285                1290                1295
Thr Leu Tyr Asn Asp Cys Trp Arg Asp Asn Trp Thr Tyr Ile Met Asp
            1300                1305                1310
Asn Gly Ile Ala Pro Leu Leu Ile Gly Glu Trp Gly Gly Tyr Leu Asp
        1315                1320                1325
Gly Gly Asp Asn Glu Lys Trp Met Thr Tyr Leu Arg Asp Tyr Ile Ile
    1330                1335                1340
Glu Asn His Ile His His Thr Phe Trp Cys Tyr Asn Ala Asn Ser Gly
1345                1350                1355                136
Asp Thr Gly Gly Leu Val Gly Tyr Asp Phe Ser Thr Trp Asp Glu Gln
                1365                1370                1375
Lys Tyr Asn Phe Leu Lys Pro Ala Leu Trp Gln Asp Ser Lys Gly Arg
            1380                1385                1390
Phe Val Gly Leu Asp His Lys Arg Pro Leu Gly Thr Asn Gly Lys Asn
        1395                1400                1405
Ile Asn Ile Thr Ile Tyr Tyr Gln Asn Gly Glu Lys Pro Pro Val Pro
    1410                1415                1420
Lys Asn
1425
```

(2) INFORMATION FOR SEQ ID NO:44:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1751 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

```
Met Gln Glu Met Lys Ala Ile Lys Arg Val Val Ser Ile Thr Ala Leu
 1               5                  10                  15
Leu Val Leu Thr Leu Ser Leu Cys Phe Pro Gly Ile Met Pro Val Lys
            20                  25                  30
Ala Tyr Ala Gly Gly Thr Tyr Asn Tyr Gly Glu Ala Leu Gln Lys Thr
        35                  40                  45
Ile Met Phe Tyr Glu Phe Gln Met Ser Gly Lys Leu Pro Ser Trp Val
    50                  55                  60
Arg Asn Asn Trp Arg Gly Asp Ser Gly Leu Asp Asp Gly Lys Asp Val
65                  70                  75                  80
Gly Leu Asp Leu Thr Gly Gly Trp His Asp Ala Gly Asp His Val Lys
                85                  90                  95
Phe Asn Leu Pro Met Ser Tyr Ser Ala Ser Met Leu Gly Trp Ala Val
            100                 105                 110
Tyr Glu Tyr Lys Asp Ala Phe Val Lys Ser Lys Gln Leu Glu His Ile
```

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                115                 120                 125
Leu Asn Gln Ile Glu Trp Ala Asn Asp Tyr Phe Val Lys Cys His Pro
    130                 135                 140
Ser Lys Tyr Val Tyr Tyr Tyr Gln Val Gly Asp Pro Thr Val Asp His
145                 150                 155                 160
Asn Phe Trp Gly Pro Ala Glu Val Met Gln Met Lys Arg Pro Ala Tyr
                165                 170                 175
Lys Cys Asp Leu Ser Asn Pro Ala Ser Ser Val Val Ala Glu Thr Ala
            180                 185                 190
Ala Ser Leu Ala Val Ala Ser Val Val Ile Lys Glu Arg Asn Ser Gln
        195                 200                 205
Lys Ala Ala Ser Tyr Leu Gln His Ala Lys Asp Leu Phe Glu Phe Ala
    210                 215                 220
Asp Thr Thr Arg Ser Asp Ala Gly Tyr Thr Ala Ala Thr Gly Phe Tyr
225                 230                 235                 240
Thr Ser Gly Gly Phe Ile Asp Asp Leu Gly Trp Ala Ala Val Trp Leu
                245                 250                 255
Tyr Ile Ala Thr Asn Asp Ser Ser Tyr Leu Thr Lys Ala Glu Glu Leu
            260                 265                 270
Met Ser Glu Tyr Ala Asn Gly Thr Asn Thr Trp Thr Gln Cys Trp Asp
        275                 280                 285
Asp Val Arg Tyr Gly Thr Leu Ile Met Leu Ala Lys Ile Thr Gly Lys
    290                 295                 300
Glu Leu Tyr Lys Gly Ala Val Glu Arg Asn Leu Asp His Trp Thr Asp
305                 310                 315                 320
Arg Ile Thr Tyr Thr Pro Lys Gly Met Ala Tyr Leu Thr Gly Trp Gly
                325                 330                 335
Ser Leu Arg Tyr Ala Thr Thr Ala Ala Phe Leu Ala Cys Val Tyr Ala
            340                 345                 350
Asp Trp Ser Gly Cys Asp Ser Asn Lys Lys Thr Lys Tyr Leu Asn Phe
        355                 360                 365
Ala Lys Ser Gln Ile Asp Tyr Ala Leu Gly Ser Thr Gly Arg Ser Phe
    370                 375                 380
Val Val Gly Phe Gly Thr Asn Tyr Pro Gln His Pro His His Arg Asn
385                 390                 395                 400
Ala His Ser Ser Trp Ala Asn Ser Met Lys Ile Pro Glu Tyr His Arg
                405                 410                 415
His Ile Leu Tyr Gly Ala Leu Val Gly Gly Pro Gly Ser Asp Asp Ser
            420                 425                 430
Tyr Asn Asp Asp Ile Thr Asp Tyr Val Gln Asn Glu Val Ala Cys Asp
        435                 440                 445
Tyr Asn Ala Gly Ile Val Gly Ala Leu Ala Lys Met Tyr Gln Leu Tyr
    450                 455                 460
Gly Gly Glu Pro Ile Asp Asp Phe Lys Ala Ile Glu Thr Pro Thr Asn
465                 470                 475                 480
Asp Glu Ile Phe Val Glu Ser Lys Phe Gly Asn Ser Gln Gly Pro Asn
                485                 490                 495
Tyr Thr Glu Val Ile Ser Tyr Ile Tyr Asn Arg Thr Gly Trp Pro Pro
            500                 505                 510
Arg Val Thr Asp Lys Leu Ser Phe Lys Tyr Phe Ile Asp Leu Thr Glu
        515                 520                 525
Leu Ile Gln Ala Gly Tyr Ser Pro Asp Val Val Lys Val Asp Thr Tyr
    530                 535                 540
Tyr Ile Glu Gly Gly Lys Ile Ser Gly Pro Tyr Val Trp Asp Lys Asn
545                 550                 555                 560
Arg Asn Ile Tyr Tyr Val Leu Val Asp Phe Ser Gly Thr Lys Ile Tyr
                565                 570                 575
Pro Gly Gly Glu Val Glu His Lys Lys Gln Ala Gln Phe Lys Ile Ser
            580                 585                 590
Val Pro Gln Gly Tyr Pro Trp Asp Pro Thr Asn Asp Pro Ser Tyr Lys
        595                 600                 605
```

```
Gly Leu Thr Ser Gln Leu Glu Lys Asn Lys Tyr Ile Ala Ala Tyr Asp
    610                 615                 620
Asn Asn Asn Leu Val Trp Gly Leu Glu Pro Gly Ala Ala Thr Ser Thr
625                 630                 635                 640
Pro Ala Pro Thr Ser Thr Pro Thr Pro Thr Pro Thr Pro Thr Pro Thr
                645                 650                 655
Val Thr Ala Thr Pro Thr Pro Thr Pro Thr Pro Thr Pro Thr Gly Ser
            660                 665                 670
Pro Gly Thr Gly Ser Gly Val Lys Val Leu Tyr Lys Asn Asn Glu Thr
        675                 680                 685
Ser Ala Ser Thr Gly Ser Ile Arg Pro Trp Phe Lys Ile Val Asn Gly
    690                 695                 700
Gly Ser Ser Ser Val Asp Leu Ser Arg Val Lys Ile Arg Tyr Trp Tyr
705                 710                 715                 720
Thr Val Asp Gly Asp Lys Pro Gln Ser Ala Val Cys Asp Trp Ala Gln
                725                 730                 735
Ile Gly Ala Ser Asn Val Thr Phe Asn Phe Val Lys Leu Ser Ser Gly
            740                 745                 750
Val Ser Gly Ala Asp Tyr Tyr Leu Glu Val Gly Phe Ser Ser Gly Ala
        755                 760                 765
Gly Gln Leu Gln Pro Gly Lys Asp Thr Gly Asp Ile Gln Val Arg Phe
    770                 775                 780
Asn Lys Asn Asp Trp Ser Asn Tyr Asn Gln Ala Asp Asp Trp Ser Trp
785                 790                 795                 800
Leu Gln Ser Met Thr Asn Tyr Gly Glu Asn Ala Lys Val Thr Leu Tyr
                805                 810                 815
Val Asp Gly Val Leu Val Trp Gly Gln Glu Pro Gly Gly Ala Thr Pro
            820                 825                 830
Ala Pro Thr Ser Thr Ala Thr Pro Thr Pro Thr Pro Thr Ala Thr Pro
        835                 840                 845
Thr Pro Thr Pro Thr Pro Thr Pro Thr Pro Thr Val Ser Ala Thr Pro
    850                 855                 860
Thr Pro Ala Pro Thr Ala Ser Pro Val Gly Gly Ser Tyr Trp Thr Pro
865                 870                 875                 880
Ser Glu Ser Tyr Gly Ala Leu Lys Val Trp Tyr Ala Asn Gly Asn Leu
                885                 890                 895
Ser Ser Pro Thr Asn Val Leu Asn Pro Lys Ile Lys Ile Glu Asn Val
            900                 905                 910
Gly Thr Thr Ala Val Asp Leu Ser Arg Val Lys Val Arg Tyr Trp Tyr
        915                 920                 925
Thr Ile Asp Gly Glu Ala Thr Gln Ser Val Ser Val Ala Ser Ser Ile
    930                 935                 940
Asn Pro Ala Tyr Ile Asp Val Lys Phe Val Lys Leu Gly Ala Asn Ala
945                 950                 955                 960
Gly Gly Ala Asp Tyr Tyr Val Glu Ile Gly Phe Lys Ser Gly Ala Gly
                965                 970                 975
Val Leu Ala Ala Gly Gln Ser Thr Lys Glu Ile Arg Leu Ser Ile Gln
            980                 985                 990
Lys Gly Ser Gly Ser Tyr Asn Gln Ser Asn Asp Tyr Ser Val Arg Ser
        995                 1000                1005
Ala Asn Ser Tyr Ile Glu Asn Glu Lys Val Thr Gly Tyr Ile Asp Asp
    1010                1015                1020
Val Leu Val Trp Gly Arg Glu Pro Gly Arg Asn Ala Gln Ile Lys Val
1025                1030                1035                104
Trp Tyr Ala Asn Gly Asn Leu Gly Ser Met Thr Asn Val Leu Asn Pro
                1045                1050                1055
Lys Ile Lys Ile Glu Asn Val Gly Thr Thr Ala Val Asp Leu Ser Arg
            1060                1065                1070
Val Lys Val Arg Tyr Trp Tyr Thr Ile Asp Gly Glu Ala Thr Gln Ser
        1075                1080                1085
Val Ser Val Thr Ser Ser Ile Asn Pro Ala Tyr Ile Asp Val Lys Phe
```

```
    1090                1095                1100
Val Lys Leu Gly Ala Asn Ala Gly Gly Ala Asp Tyr Tyr Val Glu Ile
1105                1110                1115                112
Gly Phe Lys Ser Gly Ala Gly Val Leu Ala Ala Gly Gln Ser Thr Lys
                1125                1130                1135
Glu Ile Arg Leu Ser Ile Gln Lys Gly Ser Gly Ser Tyr Asn Gln Ser
            1140                1145                1150
Asn Asp Tyr Ser Val Arg Ser Ala Thr Gly Tyr Ile Glu Asn Glu Lys
        1155                1160                1165
Val Thr Gly Tyr Ile Asp Gly Ala Ile Val Trp Gly Arg Glu Pro Ser
    1170                1175                1180
Arg Gly Thr Lys Pro Ala Gly Gly Val Thr Pro Thr Pro Ala Pro Thr
1185                1190                1195                120
Pro Thr Ser Thr Pro Thr Pro Thr Pro Thr Thr Thr Pro Thr Pro Thr
                1205                1210                1215
Pro Thr Val Thr Val Thr Pro Thr Pro Thr Pro Ala Val Thr Pro Asp
            1220                1225                1230
Val Lys Ile Ser Ile Asp Thr Ser Arg Gly Arg Thr Lys Ile Ser Pro
        1235                1240                1245
Tyr Ile Tyr Gly Ala Asn Gln Asp Ile Gln Gly Val Val His Pro Ala
    1250                1255                1260
Arg Arg Leu Gly Gly Asn Arg Leu Thr Gly Tyr Asn Trp Glu Asn Asn
1265                1270                1275                128
Met Ser Asn Ala Gly Ser Asp Trp Tyr His Ser Ser Asp Asp Tyr Met
                1285                1290                1295
Cys Tyr Ile Met Gly Ile Thr Gly Asn Asp Lys Asn Val Pro Ala Ala
            1300                1305                1310
Val Val Ser Lys Phe His Glu Gln Ser Ile Lys Gln Asn Ala Tyr Ser
        1315                1320                1325
Ala Ile Thr Leu Gln Met Val Gly Tyr Val Ala Lys Asp Gly Asn Gly
    1330                1335                1340
Thr Val Ser Glu Ser Glu Thr Ala Pro Ser Pro Arg Trp Ala Glu Val
1345                1350                1355                136
Lys Phe Lys Lys Asp Gly Ala Leu Ser Leu Gln Pro Asp Val Asn Asp
                1365                1370                1375
Asn Tyr Val Tyr Met Asp Glu Phe Ile Asn Tyr Leu Ile Asn Lys Tyr
            1380                1385                1390
Gly Arg Ser Ser Ser Ala Thr Gly Ile Lys Gly Tyr Ile Leu Asp Asn
        1395                1400                1405
Glu Pro Asp Leu Trp Phe Thr Thr His Pro Arg Ile His Pro Gln Lys
    1410                1415                1420
Val Thr Cys Ser Glu Leu Ile Asn Lys Ser Val Glu Leu Ala Lys Val
1425                1430                1435                144
Ile Lys Thr Leu Asp Pro Asp Ala Glu Ile Phe Gly Pro Ala Ser Tyr
                1445                1450                1455
Gly Phe Val Gly Tyr Leu Thr Leu Gln Asp Ala Pro Asp Trp Asn Gln
            1460                1465                1470
Val Lys Gly Asn His Arg Trp Phe Leu Ser Trp Tyr Leu Glu Gln Met
        1475                1480                1485
Lys Lys Ala Ser Asp Ser Phe Gly Lys Arg Leu Leu Asp Val Leu Asp
    1490                1495                1500
Ile His Trp Tyr Pro Glu Ala Gln Val Gly Gly Val Arg Ile Cys Phe
1505                1510                1515                152
Asp Gly Glu Asn Ser Thr Ser Arg Asp Val Ala Ile Ala Arg Met Gln
                1525                1530                1535
Ala Pro Arg Thr Leu Trp Asp Pro Thr Tyr Lys Thr Thr Gln Lys Gly
            1540                1545                1550
Gln Ile Thr Ala Gly Glu Asn Ser Trp Ile Asn Gln Trp Phe Pro Glu
        1555                1560                1565
Tyr Leu Pro Leu Leu Pro Asn Ile Lys Ala Asp Ile Asp Lys Tyr Tyr
    1570                1575                1580
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Pro Gly Thr Lys Leu Ala Ile Thr Glu Phe Asp Tyr Gly Gly Lys Asp
1585                1590                1595                160
His Ile Ser Gly Gly Ile Ala Leu Ala Asp Val Leu Gly Ile Phe Gly
                1605                1610                1615
Lys Tyr Gly Val Tyr Met Ala Ala Arg Trp Gly Asp Ser Gly Ser Tyr
            1620                1625                1630
Ala Gln Ala Ala Tyr Asn Ile Tyr Leu Asn Tyr Asp Gly Lys Gly Ser
        1635                1640                1645
Arg Tyr Gly Ser Thr Cys Val Ser Ala Glu Thr Thr Asp Val Glu Asn
    1650                1655                1660
Met Pro Val Tyr Ala Ser Ile Glu Gly Glu Asp Asp Ser Thr Val His
1665                1670                1675                168
Ile Ile Leu Ile Asn Arg Asn Tyr Asp Arg Lys Leu Lys Ala Glu Ile
                1685                1690                1695
Lys Met Asn Asn Thr Arg Val Tyr Thr Gly Gly Glu Ile Tyr Gly Phe
            1700                1705                1710
Asp Ser Thr Ser Ser Gln Ile Arg Lys Met Gly Val Leu Ser Asn Ile
        1715                1720                1725
Gln Asn Asn Thr Ile Thr Ile Glu Val Pro Asn Leu Thr Val Tyr His
    1730                1735                1740
Ile Val Leu Thr Ser Ser Lys
1745                1750
```

(2) INFORMATION FOR SEQ ID NO:45:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

```
GCGTGGTATG CAATATAC                                                  18
```

(2) INFORMATION FOR SEQ ID NO:46:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2029 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

```
ATGGGAAGTG GTGTGAAGGT ACTGTACAAG AACAATGAGA CAAGTGCGAG CACAGGTTCT     60
ATAAGGCCGT GGTTTAAGAT AGTGAATGGA GGCAGCAGCA GTGTTGATCT TAGCAGGGTT    120
AAGATAAGAT ACTGGTACAC AGTGGATGGT GACAAGCCAC AGAGTGCGGT ATGTGACTGG    180
GCACAGATAG GGGCAAGCAA TGTGACATTC AATTTTGTGA AGCTTAGCAG CGGAGTGAGT    240
GGAGCGGATT ATTACCTGGA GGTAGGATTT AGCAGTGGAG CTGGGCAGTT GCAGCCTGGT    300
AAGGACACAG GGGATATACA GGTAAGGTTT AACAAGAATG ACTGGAGCAA TTACAATCAG    360
GCAGACGACT GGTCATGGTT GCAGAGCATG ACGAATTATG GAGAGAATGC GAAGGTGACG    420
CTGTATGTAG ATGGTGTTCT GGTATGGGGG CAGGAGCCGG GAGGAGCGGT GACCCCAACT    480
TCTACACCCA CACCGGTTTC ATCATCCACT CCTACACCAA CAGCAACGCC AACACCTACA    540
CCTTCTATCA CGATAACACC AGCGCCAACT GCAACACCCA CTCCGACTCC TTCTGTCACA    600
GATGATACAA ATGATGATTG GTTATTTGCG CAGGGTAACA AAATAGTCGA CAAGGATGGC    660
AAACCTGTAT GGTTAACAGG AGTTAATTGG TTTGGATTTA ATACAGGAAC GAATGTGTTT    720
GATGGTGTGT GGAGTTGTAA TCTTAAAAGT GCATTAGCTG AGATTGCAAA CAGAGGATTT    780
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AATTTGCTAA GAGTACCGAT TTCAGCAGAG CTGATTTTGA ATTGGTCGAA AGGAATTTAT    840
CCAAAACCAA ATATCAATTA TTATGTTAAC CCTGAGTTAG AAGGTCTGAC GAGTTTAGAG    900
GTATTTGATT TTGTAGTAAA AACATGCAAA GAAGTTGGAC TGAAAATTAT GTTGGATATT    960
CATAGTGCAA AAACTGATGC GATGGGGCAT ATATATCCGG TATGGTATAC AGATACTATA   1020
ACGCCAGAAG ATTATTATAA AGCATGTGAA TGGATCACAG AGAGATATAA AAATGATGAT   1080
ACAATTGTAG CATTTGATTT GAAGAATGAG CCACATGGTA AACCATGGCA AGATAGTGTT   1140
TTTGCAAAAT GGGACAATTC AACAGATATT AACAACTGGA AATATGCAGC TGAGACCTGT   1200
GCGAAGAGAA TACTTGCAAA AAATCCAAAC ATGTTAATAG TAATTGAAGG AATAGAAGCT   1260
TATCCAAAAG ATGATGTTAC GTGGACTTCT AAATCATCAA GTGACTATTA TTCTACCTGG   1320
TGGGGCGGCA ACTTACGGGG TGTTAAAAAG TATCCAATAA ACCTTGGACA GTATCAGAAC   1380
AAAGTGGTTT ATTCACCACA TGATTATGGA CCATTGGTTT ACCAGCAACC CTGGTTTTAT   1440
CCTGGATTTA CCAAAGATAC GCTTTACAAT GATTGCTGGA GGGATAATTG GACTTATATT   1500
ATGGATAATG GGATAGCTCC GTTGCTCATT GGTGAATGGG GTGGTTACTT AGATGGTGGC   1560
GATAATGAAA AGTGGATGAC TTATTTGAGA GATTATATTA TAGAAAACCA TATTCATCAT   1620
ACATTCTGGT GTTACAATGC AAATTCTGGT GATACTGGAG GATTGGTGGG ATATGATTTT   1680
TCGACGTGGG ATGAACAGAA GTACAATTTC TTAAAACCAG CTTTATGGCA GGATAGTAAA   1740
GGAAGATTTG TTGGGCTTGA TCACAAGAGA CCACTGGGTA CAAATGGGAA GAATATAAAT   1800
ATAACTATTT ATTACCAGAA CGGTGAAAAA CCGCCTGTCC CAAAGAATTA ATAAATGGAT   1860
CCGGCTGCTA ACAAAGCCCG AAAGGAAGCT GAGTTGGCTG CTGCCACCGC TGAGCAATAA   1920
CTAGCATAAC CCCTTGGGGC CTCTAAACGG GTCTTGAGGG GTTTTTTGCT GAAAGGAGGA   1980
ACTATATCCG GATATCCACA GGACGGGTGT GGTCGCCATG ATCGCGTAG               2029
```

(2) INFORMATION FOR SEQ ID NO:47:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 616 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

```
Met Gly Ser Gly Val Lys Val Leu Tyr Lys Asn Asn Glu Thr Ser Ala
 1               5                  10                  15
Ser Thr Gly Ser Ile Arg Pro Trp Phe Lys Ile Val Asn Gly Gly Ser
            20                  25                  30
Ser Ser Val Asp Leu Ser Arg Val Lys Ile Arg Tyr Trp Tyr Thr Val
        35                  40                  45
Asp Gly Asp Lys Pro Gln Ser Ala Val Cys Asp Trp Ala Gln Ile Gly
    50                  55                  60
Ala Ser Asn Val Thr Phe Asn Phe Val Lys Leu Ser Ser Gly Val Ser
65                  70                  75                  80
Gly Ala Asp Tyr Tyr Leu Glu Val Gly Phe Ser Ser Gly Ala Gly Gln
                85                  90                  95
Leu Gln Pro Gly Lys Asp Thr Gly Asp Ile Gln Val Arg Phe Asn Lys
            100                 105                 110
Asn Asp Trp Ser Asn Tyr Asn Gln Ala Asp Asp Trp Ser Trp Leu Gln
        115                 120                 125
Ser Met Thr Asn Tyr Gly Glu Asn Ala Lys Val Thr Leu Tyr Val Asp
    130                 135                 140
Gly Val Leu Val Trp Gly Gln Glu Pro Gly Gly Ala Val Thr Pro Thr
145                 150                 155                 160
Ser Thr Pro Thr Pro Val Ser Ser Ser Thr Pro Thr Pro Thr Ala Thr
                165                 170                 175
Pro Thr Pro Thr Pro Ser Ile Thr Ile Thr Pro Ala Pro Thr Ala Thr
            180                 185                 190
Pro Thr Pro Thr Pro Ser Val Thr Asp Asp Thr Asn Asp Asp Trp Leu
        195                 200                 205
Phe Ala Gln Gly Asn Lys Ile Val Asp Lys Asp Gly Lys Pro Val Trp
```

```
    210                 215                 220
Leu Thr Gly Val Asn Trp Phe Gly Phe Asn Thr Gly Thr Asn Val Phe
225                 230                 235                 240
Asp Gly Val Trp Ser Cys Asn Leu Lys Ser Ala Leu Ala Glu Ile Ala
                245                 250                 255
Asn Arg Gly Phe Asn Leu Leu Arg Val Pro Ile Ser Ala Glu Leu Ile
            260                 265                 270
Leu Asn Trp Ser Lys Gly Ile Tyr Pro Lys Pro Asn Ile Asn Tyr Tyr
        275                 280                 285
Val Asn Pro Glu Leu Glu Gly Leu Thr Ser Leu Glu Val Phe Asp Phe
    290                 295                 300
Val Val Lys Thr Cys Lys Glu Val Gly Leu Lys Ile Met Leu Asp Ile
305                 310                 315                 320
His Ser Ala Lys Thr Asp Ala Met Gly His Ile Tyr Pro Val Trp Tyr
                325                 330                 335
Thr Asp Thr Ile Thr Pro Glu Asp Tyr Tyr Lys Ala Cys Glu Trp Ile
            340                 345                 350
Thr Glu Arg Tyr Lys Asn Asp Asp Thr Ile Val Ala Phe Asp Leu Lys
        355                 360                 365
Asn Glu Pro His Gly Lys Pro Trp Gln Asp Ser Val Phe Ala Lys Trp
    370                 375                 380
Asp Asn Ser Thr Asp Ile Asn Asn Trp Lys Tyr Ala Ala Glu Thr Cys
385                 390                 395                 400
Ala Lys Arg Ile Leu Ala Lys Asn Pro Asn Met Leu Ile Val Ile Glu
                405                 410                 415
Gly Ile Glu Ala Tyr Pro Lys Asp Asp Val Thr Trp Thr Ser Lys Ser
            420                 425                 430
Ser Ser Asp Tyr Tyr Ser Thr Trp Trp Gly Gly Asn Leu Arg Gly Val
        435                 440                 445
Lys Lys Tyr Pro Ile Asn Leu Gly Gln Tyr Gln Asn Lys Val Val Tyr
    450                 455                 460
Ser Pro His Asp Tyr Gly Pro Leu Val Tyr Gln Gln Pro Trp Phe Tyr
465                 470                 475                 480
Pro Gly Phe Thr Lys Asp Thr Leu Tyr Asn Asp Cys Trp Arg Asp Asn
                485                 490                 495
Trp Thr Tyr Ile Met Asp Asn Gly Ile Ala Pro Leu Leu Ile Gly Glu
            500                 505                 510
Trp Gly Gly Tyr Leu Asp Gly Gly Asp Asn Glu Lys Trp Met Thr Tyr
        515                 520                 525
Leu Arg Asp Tyr Ile Ile Glu Asn His Ile His His Thr Phe Trp Cys
    530                 535                 540
Tyr Asn Ala Asn Ser Gly Asp Thr Gly Gly Leu Val Gly Tyr Asp Phe
545                 550                 555                 560
Ser Thr Trp Asp Glu Gln Lys Tyr Asn Phe Leu Lys Pro Ala Leu Trp
                565                 570                 575
Gln Asp Ser Lys Gly Arg Phe Val Gly Leu Asp His Lys Arg Pro Leu
            580                 585                 590
Gly Thr Asn Gly Lys Asn Ile Asn Ile Thr Ile Tyr Tyr Gln Asn Gly
        595                 600                 605
Glu Lys Pro Pro Val Pro Lys Asn
    610                 615
```

Claims

1. A DNA sequence free of native source genomic DNA and encoding a cellulase active protein comprising the (Cel B5) amino acid sequence extending from amino acid position No. A1001 through amino acid position No. P1424

or K1425 or N1426 in SEQ. ID No. 43, or the (Cel B4/5) amino acid sequence extending from amino acid position No. K635 through amino acid position No. N1426 in SEQ. ID No.43, or the (Cel E1) amino acid sequence extending from amino acid position No.Y39 through amino acid position No. D481 in SEQ. ID No. 44, or the (Cel E1/2) amino acid sequence extending from amino acid position No. Y39 through amino acid position No. G635 in SEQ. ID No. 44, or the (Cel E1/2/3) amino acid sequence extending from amino acid position No. Y39 through amino acid position No. G812 in SEQ. ID No. 44, or the (Cel E6) amino acid sequence extending from amino acid position No. V1233 through amino acid position No. K1751 in SEQ. ID No. 44, or the (stability region) amino acid sequence extending from amino acid position No. E482 through amino acid position No.G635 in SEQ. ID No. 44, or the (Cel E3/B5) amino acid sequence in SEQ. ID No. 47, or a functional equivalent of said proteins.

2. A recombinant DNA vector comprising:

a) a DNA sequence encoding a cellulase active protein according to claim 1; and
b) heterologous vector DNA.

3. A recombinant DNA expression vector according to claim 2 in which the vector DNA comprises promoter DNA operatively controlling expression of the DNA encoding the cellulase protein.

4. A recombinant DNA expression vector according to claim 3 in which said promoter DNA is heterologous DNA.

5. A recombinant DNA expression vector according to claim 3 in which the vector DNA comprises homologous promoter DNA operatively controlling expression of the DNA encoding the cellulase protein.

6. A cell transformed with an expression vector of claim 3.

7. A recombinant cellulase active protein substantially free of proteinases of native thermophilic and alkalinophilic origin and comprising the (Cel B5) amino acid sequence extending from amino acid position No. A1001 through amino acid position No. P1424 or K1425 or N1426 in SEQ. ID No. 43, or the (Cel B4/5) amino acid sequence extending from amino acid position No. K635 through amino acid position No. N1426 in SEQ. ID No.43, or the (Cel E1) amino acid sequence extending from amino acid position No.Y39 through amino acid position No. D481 in SEQ. ID No. 44, or the (Cel E1/2) amino acid sequence extending from amino acid position No. Y39 through amino acid position No. G635 in SEQ. ID No. 44, or the (Cel E1/2/3) amino acid sequence extending from amino acid position No. Y39 through amino acid position No. G812 in SEQ. ID No. 44, or the (Cel E6) amino acid sequence extending from amino acid position No. V1233 through amino acid position No. K1751 in SEQ. ID No. 44, or the (stability region) amino acid sequence extending from amino acid position No. E482 through amino acid position No.G635 in SEQ. ID No. 44, or the (Cel E3/B5) amino acid sequence in SEQ. ID No. 47, or a functional equivalent thereof.

8. A DNA sequence free of native source genomic DNA and encoding a fragment of cellulase active protein comprising the (tokcelef) nucleotide sequence of SEQ. ID No. 9, or its functional equivalent when used in the amplification of endoglucanase genes.

9. A recombinant DNA vector comprising:

a) a DNA sequence of claim 8; and
b) homologous or heterologous vector DNA.

10. A cell transformed with the expression vector of claim 9.

11. A laundry detergent composition comprising a cellulase active protein in an amount sufficient to confer anti-graying or anti-backstaining properties to the detergent composition, the cellulase active protein being selected from the group consisting of Cel B5, Cel B4/5, Cel E1, Cel E1/2, Cel E1/2/3, or Cel E6, or the protein (stability region) amino acid sequence extending from amino acid position No. E482 through amino acid position No.G635 in SEQ. ID No. 44, or Cel E3/B5, or a functional equivalent of said protein.

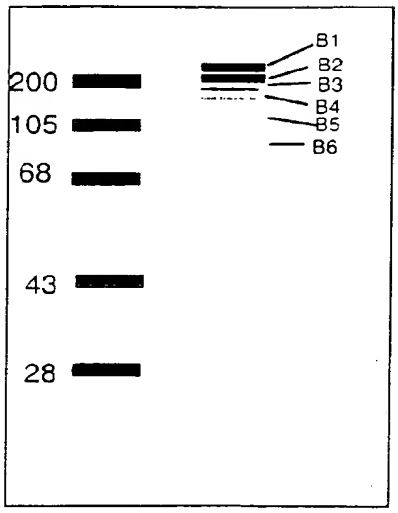
12. The method of treating cellulosic containing material to prevent or remove staining, backstaining, or graying, comprising contacting said material with an aqueous solution of laundry detergent composition containing a cellulase active protein in an amount sufficient to confer anti-staining or anti-backstaining or anti-graying properties to the

laundry detergent, the cellulase active protein being selected from the group consisting of Cel B5, Cel B4/5, Cel E1, Cel E1/2, Cel E1/2/3, or Cel E6, or the protein (stability region) amino acid sequence extending from amino acid position No. E482 through amino acid position No.G635 in SEQ. ID No. 44, or Cel E3/B5. or a functional equivalent of said protein.

13. A E.coli bacterium having the identifying characteristics of ATCC Accession Nos. 98523 or 98524 or a variant or mutant thereof which produces a cellulase active protein being selected from the group consisting of Cel B5, Cel B4/5, Cel E1, Cel E1/2, Cel E1/2/3, or Cel E6, or the protein (stability region) amino acid sequence extending from amino acid position No. E482 through amino acid position No. G635 in SEQ. ID No. 44, or Cel E3/B5, or a functional equivalent of said protein.

Figure 1

A



B

N-terminal sequence found:

B1	AAYNYGEALQKAIMFYEFXM
B2	APDWSIPSLWESYKND
B3	AAYNYGEALQ
B4	APDWSIPSLW
B5	GAYNYGEALQ
B6	GAYNY

A) A composite diagram of protein bands that contained cellulase activity from the Tok7B.1 supernatant purified on either S-sepharose or Q sepharose . The protein bands were designated B1 through B6 each of the designated bands was N-terminally sequenced.

B) The N-terminal sequence found for each band is shown above. Two seperate N -terminal sequences were identified corresponding to the N-terminus of the Cel E and Cel B genes shown in Figure 3.

Figure 2.

Blast sequence homology search with the identified N-terminal peptides shows the proteins have homology with Families 9 & 10 from Glycosyl hydrolases. Areas of homology between sequenced N-termini are shown in black backgrounds with white lettering.

Peptide No.	Amino-terminal amino acid sequence	Glycosyl Hydrolase Family based on amino acid homology comparisons
B1	AAAYNYGEALQKAIMFYEFXM	
B3	AAAYNYGEALQ	Glycosyl hydrolase
B5	GAAYNYGEALQ	Family 9
B6	GAAYNY	
B2	APDWSIPSLWESKYND	Glycosyl hydrolase
B4	APDWSIPSLW	Family 10

Figure 3.

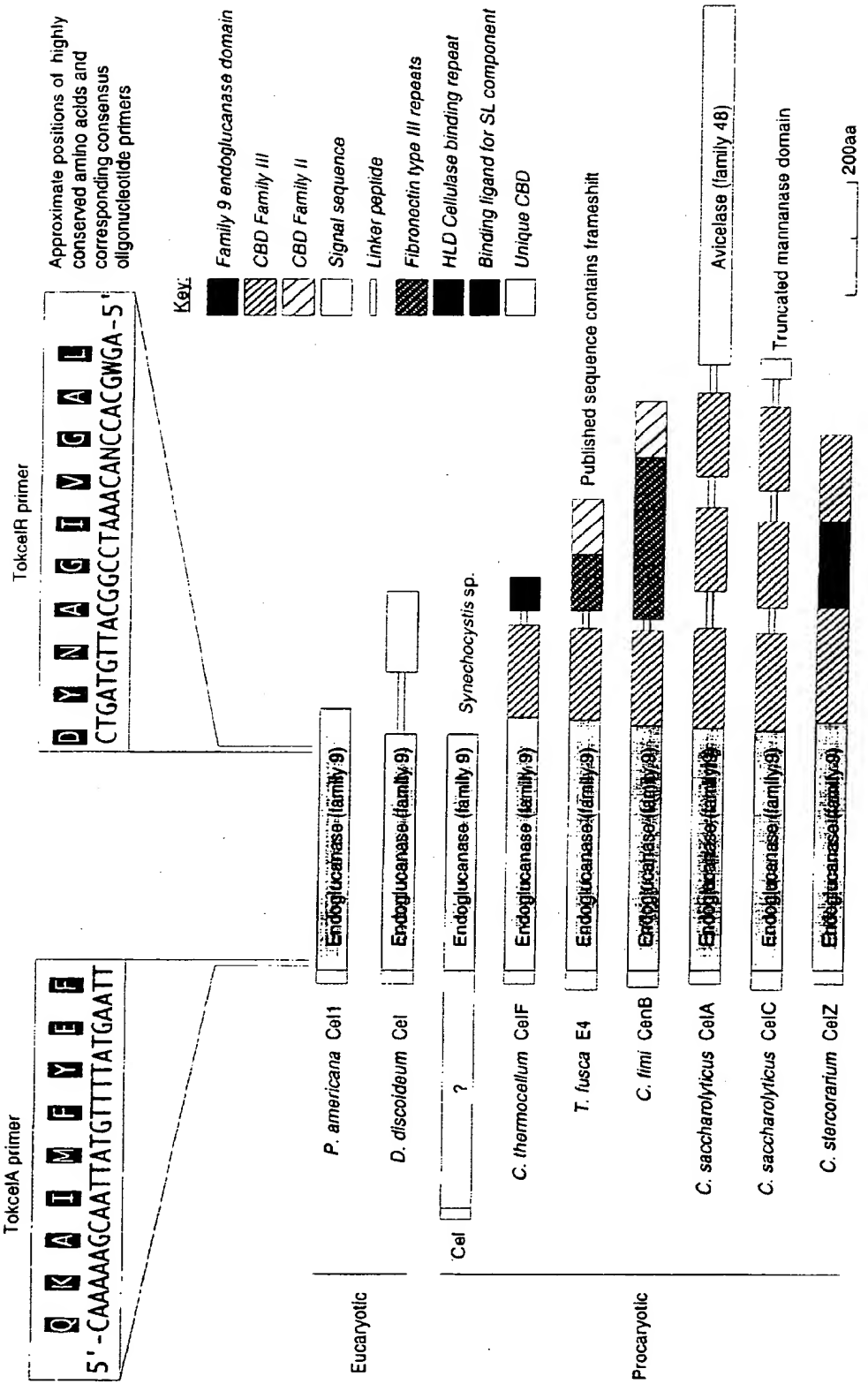


Figure 4.

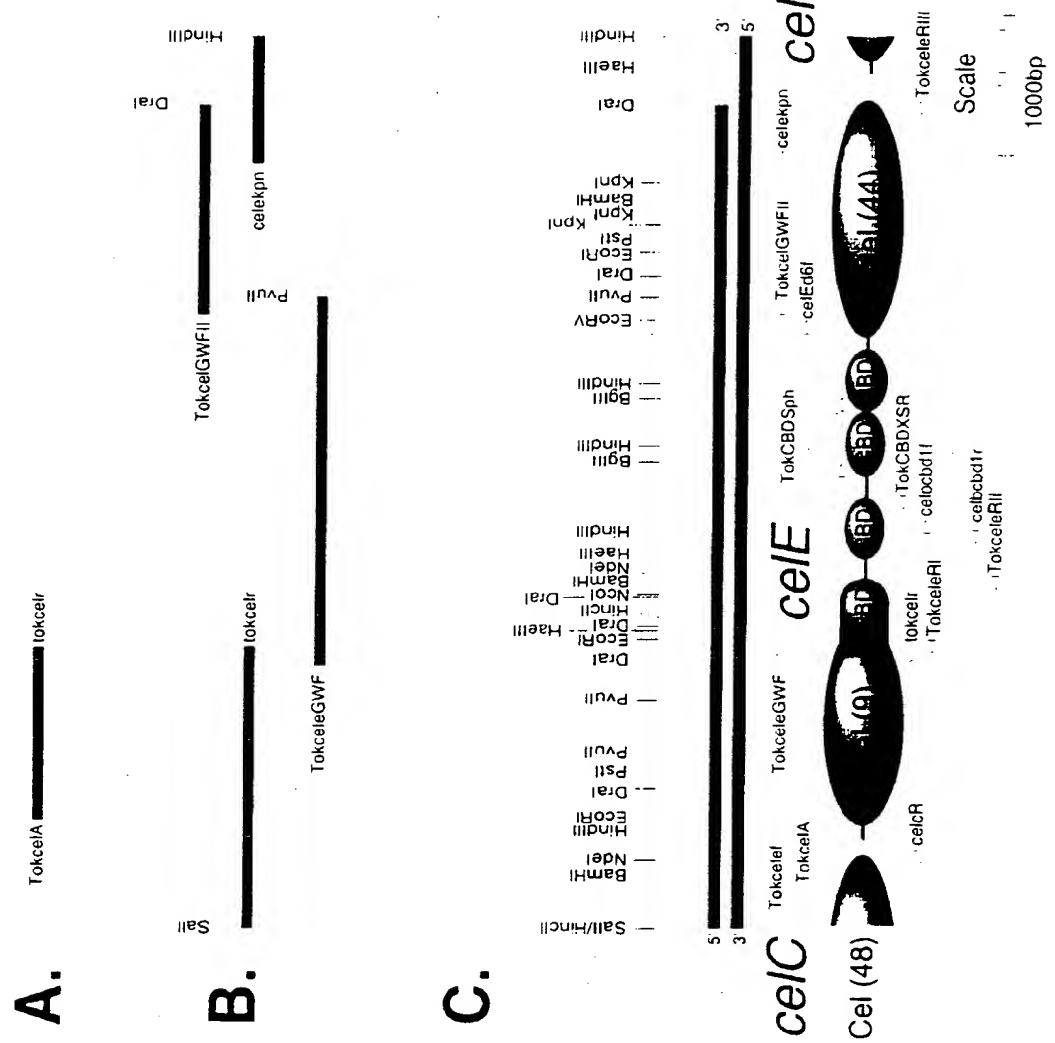


Figure 5A.

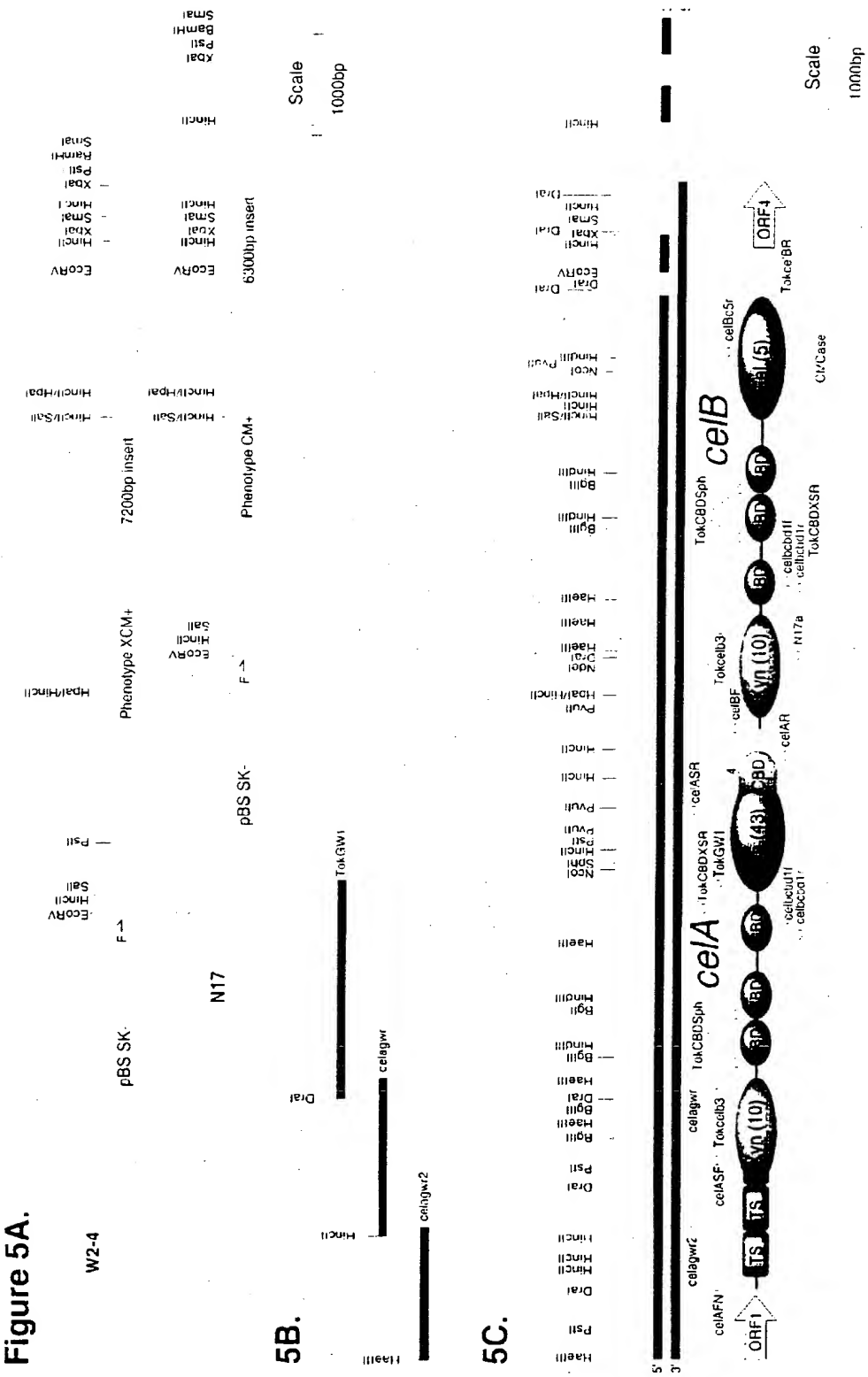


Figure 6 **Cellulase Gene Domains**

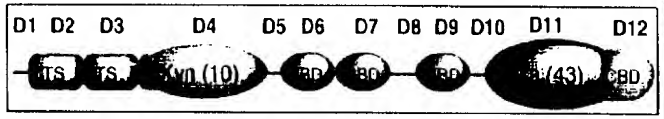
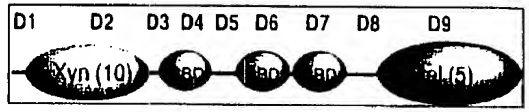
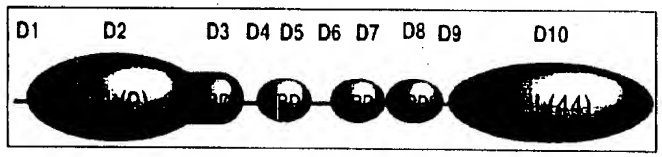
Enzyme	Protein Domain Structure	Domain	Coordinates	Function (Homology)	Reference
CelA		D1	1-33	Signal peptide	-
		D2	34-187	Thermostabilising domain	Winter
		D3	188-343	Thermostabilising domain	Winter
		D4	344-689	Endoxylanase (10)	
		D5	690-711	PT linker	Gilkes
		D6	712-877	CBD (Type III)	Tomme
		D7	878-1035	CBD (Type III)	Tomme
		D8	1036-1099	PT Linker	Gilkes
		D9	1100-1256	CBD (Type III)	Tomme
		D10	1257-1302	PT linker	Gilkes
		D11	1303-1630	Arabinofuranosidase (43)	Teo
		D12	1631-1770	CBD	Sakka
CelB		D1	1-36	Signal peptide	-
		D2	37-379	Endoxylanase (10)	
		D3	380-410	PT linker	Gilkes
		D4	411-565	CBD (Type III)	Tomme
		D5	566-616	PT linker	Gilkes
		D6	617-779	CBD (Type III)	Tomme
		D7	780-938	CBD (Type III)	Tomme
		D8	939-1007	PT linker	Gilkes
		D9	1008-1426	Endoglucanase (5)	Saul
CelE		D1	1-33	Signal Peptide	-
		D2	34-472	Endoglucanase (9)	Morris
		D3	473-639	CBD (Type II)	Tomme
		D4	640-670	PT linker	Gilkes
		D5	671-830	CBD (Type III)	Tomme
		D6	831-869	PT linker	Gilkes
		D7	870-1035	CBD (Type III)	Tomme
		D8	1036-1993	CBD (Type III)	Tomme
		D9	1194-1231	PT linker	Gilkes
		D10	1232-1751	Endoglucanase (44)	Gibbs

Figure 7A.

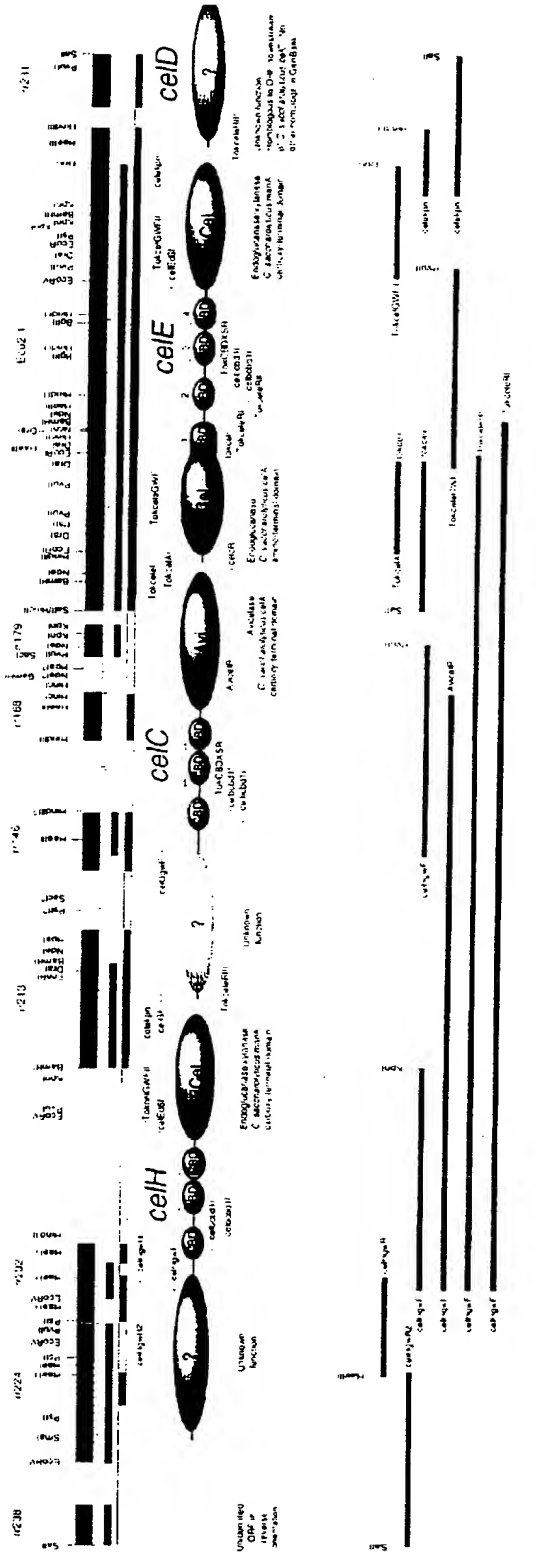


Figure 7B.

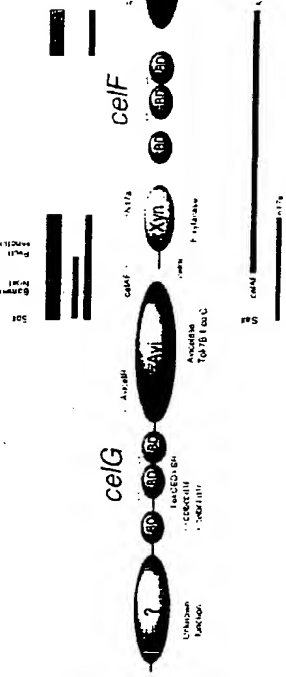


Figure 8.

Tok7B.1 CBD-catalytic domain PCR products expressed from pJLA602

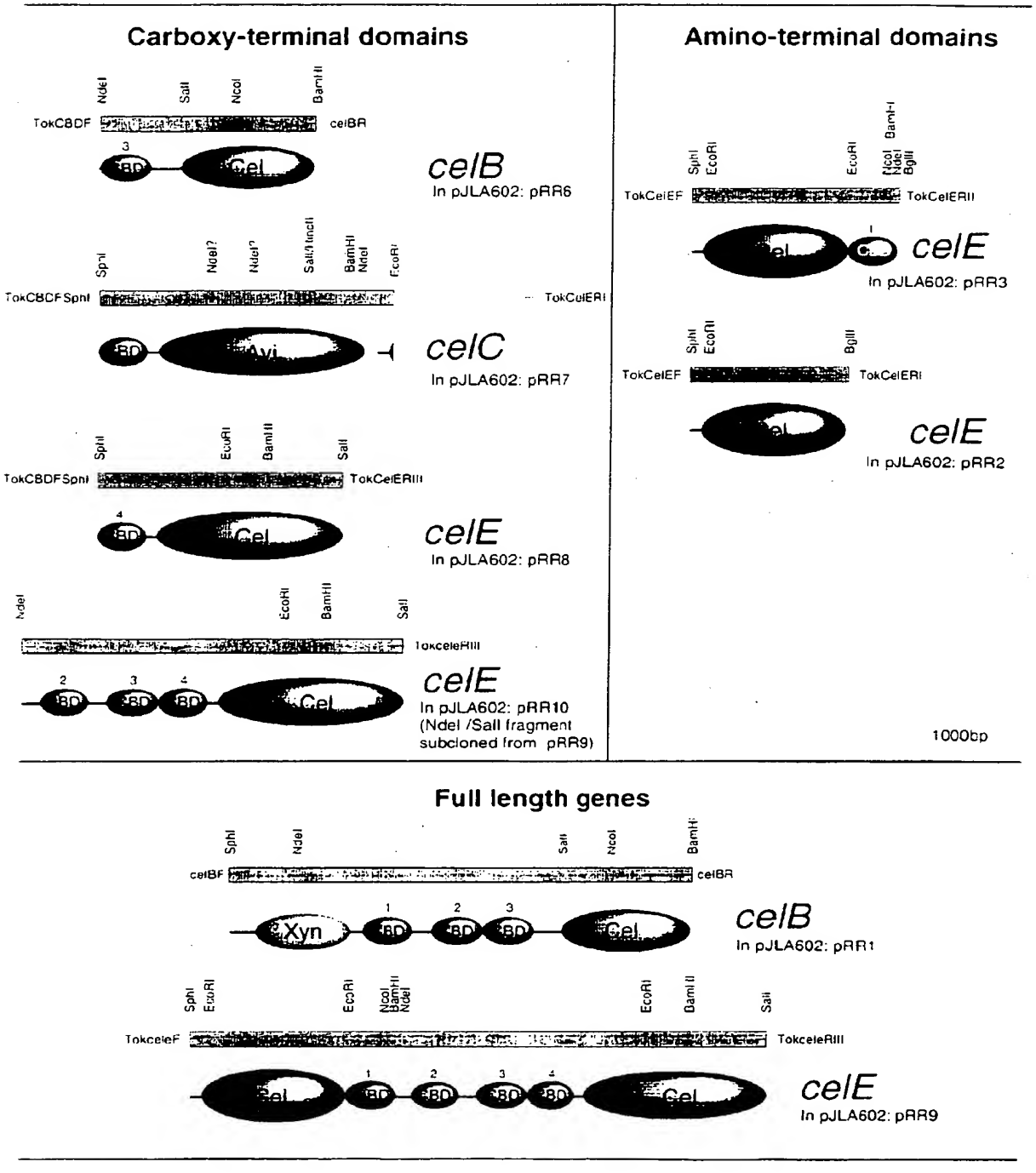


Figure 9.

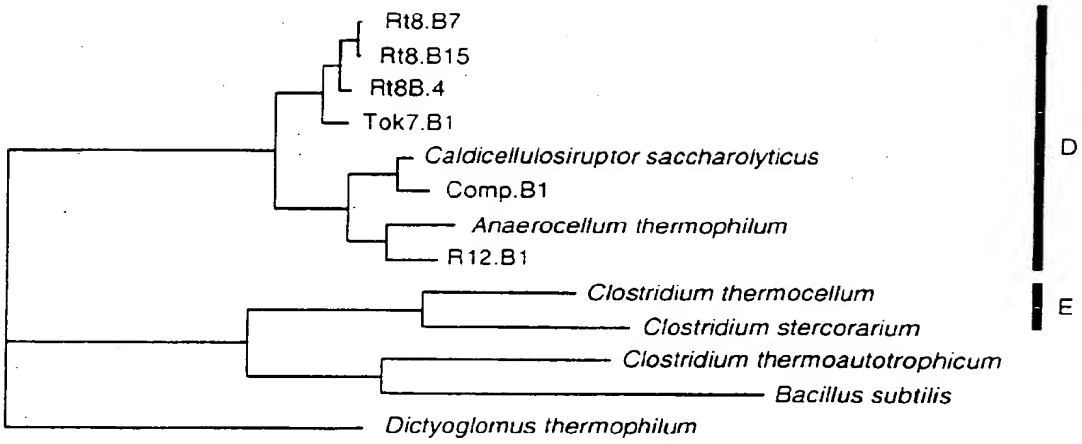


FIGURE 10

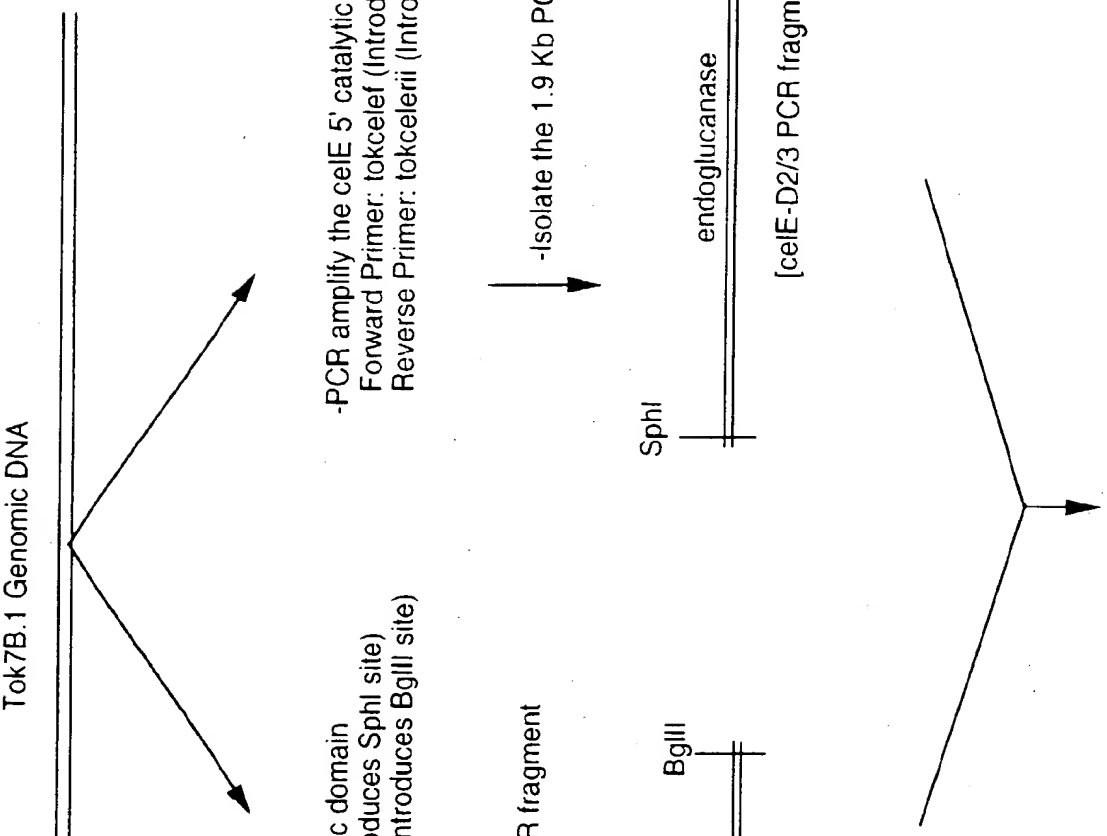


FIGURE 11

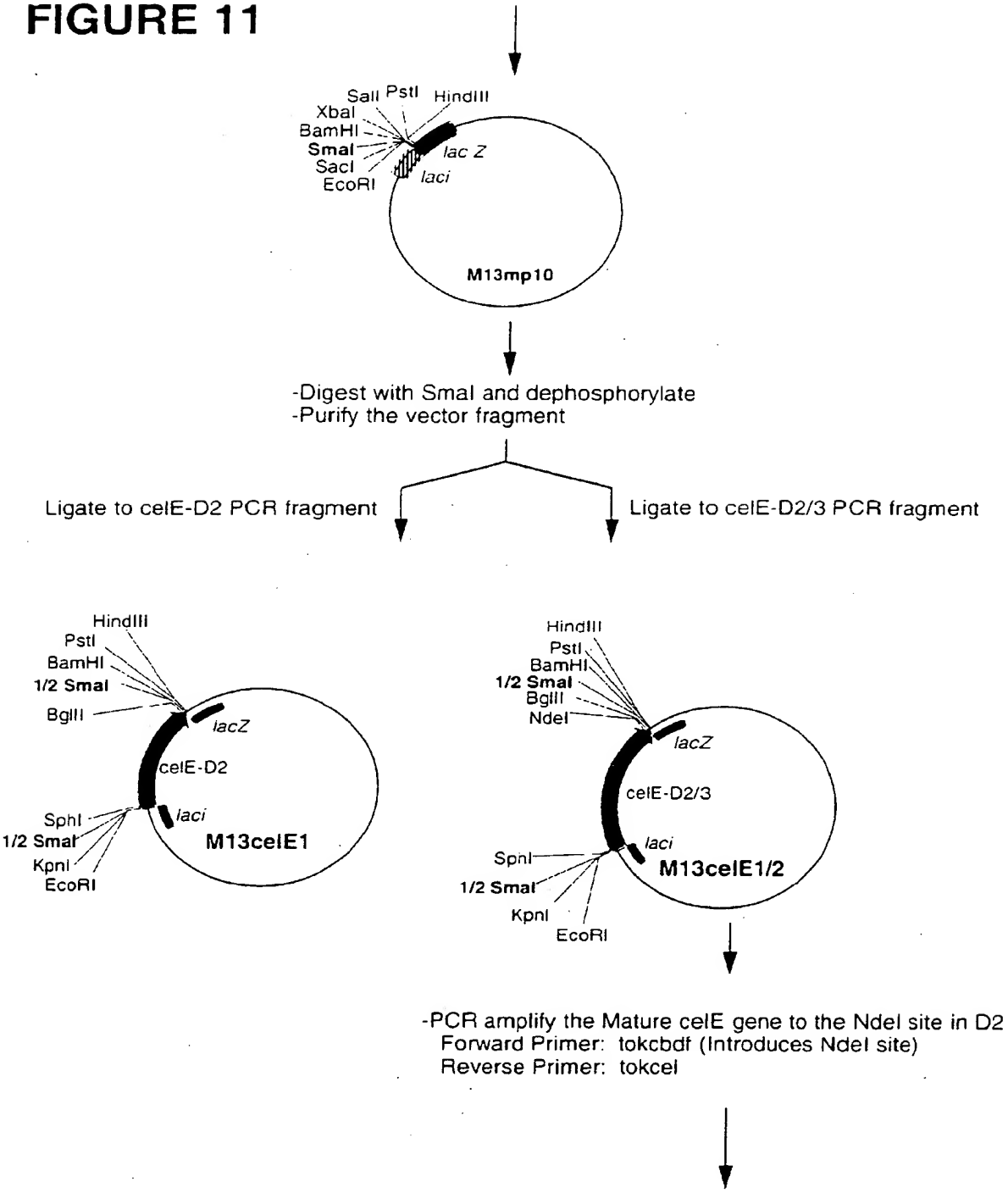


FIGURE 12

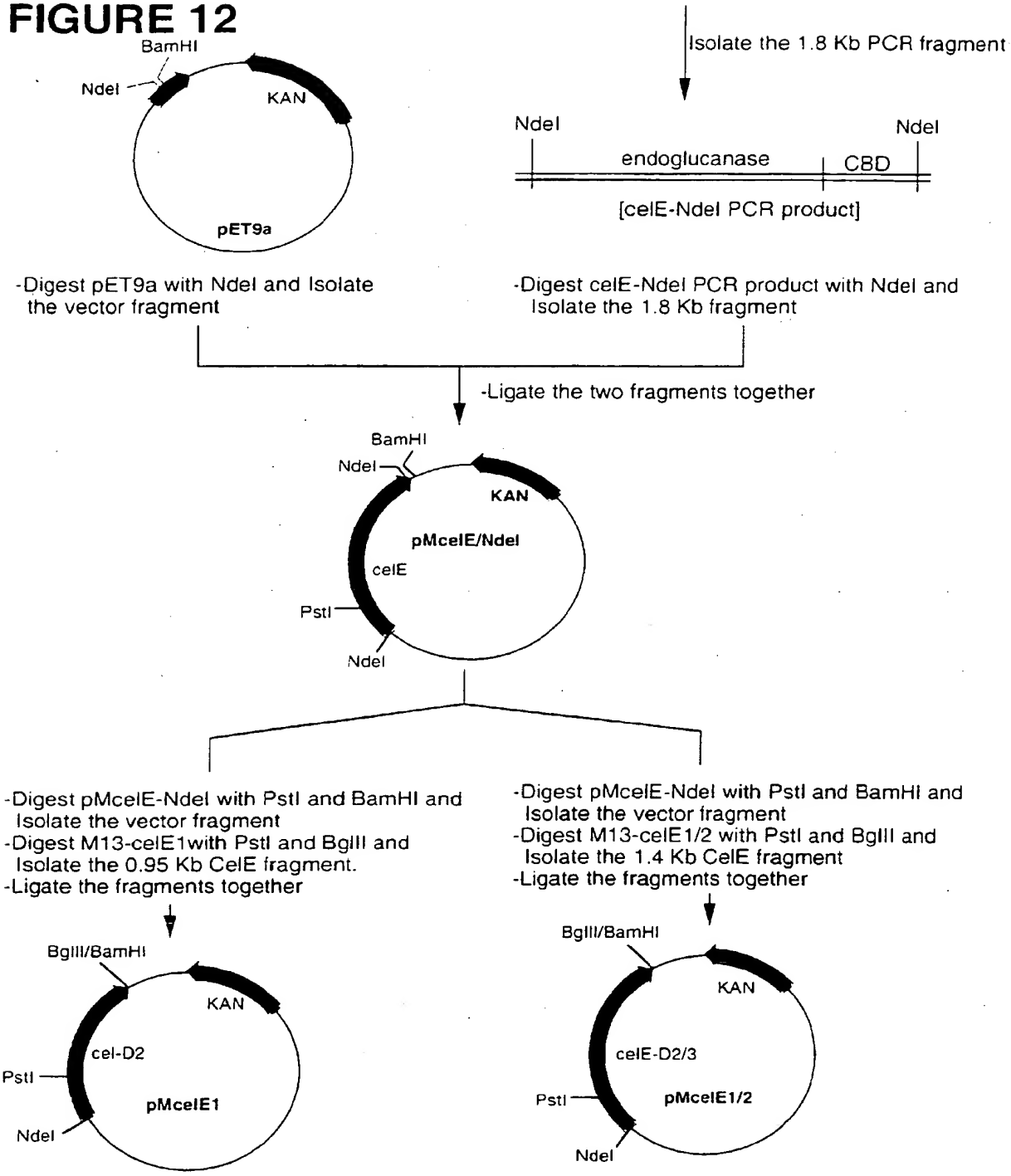


Figure 13

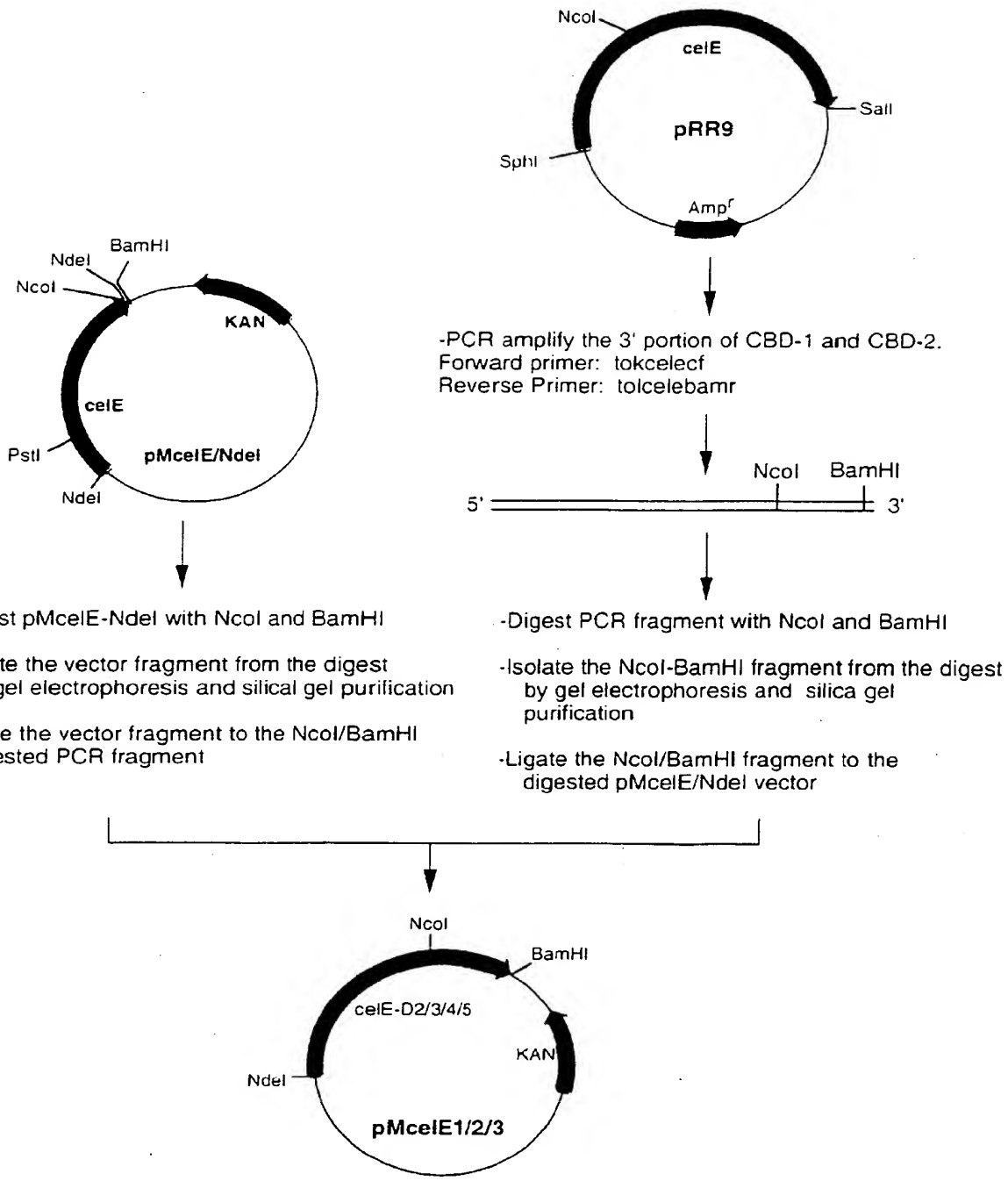


FIGURE 14

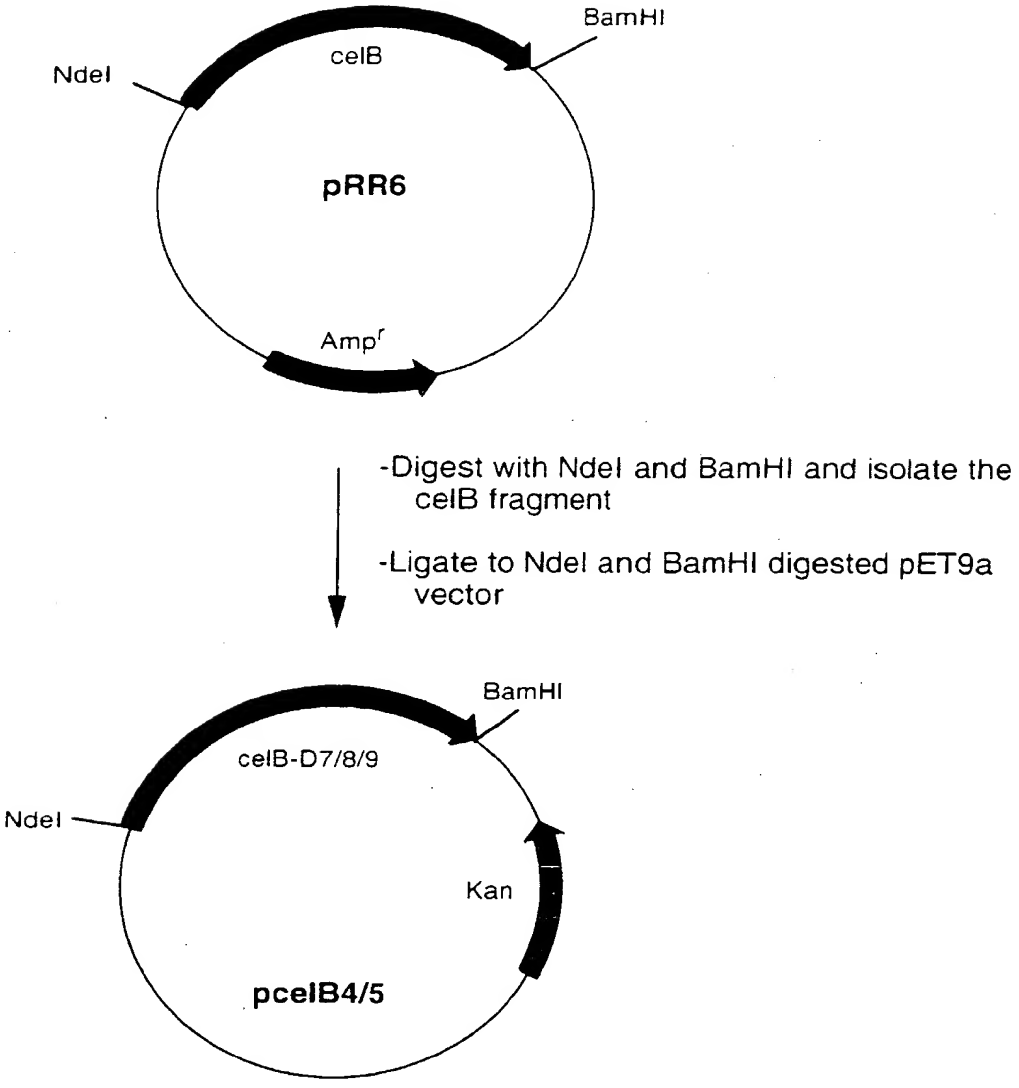


Figure 14A

Construction of pcelE3/B5

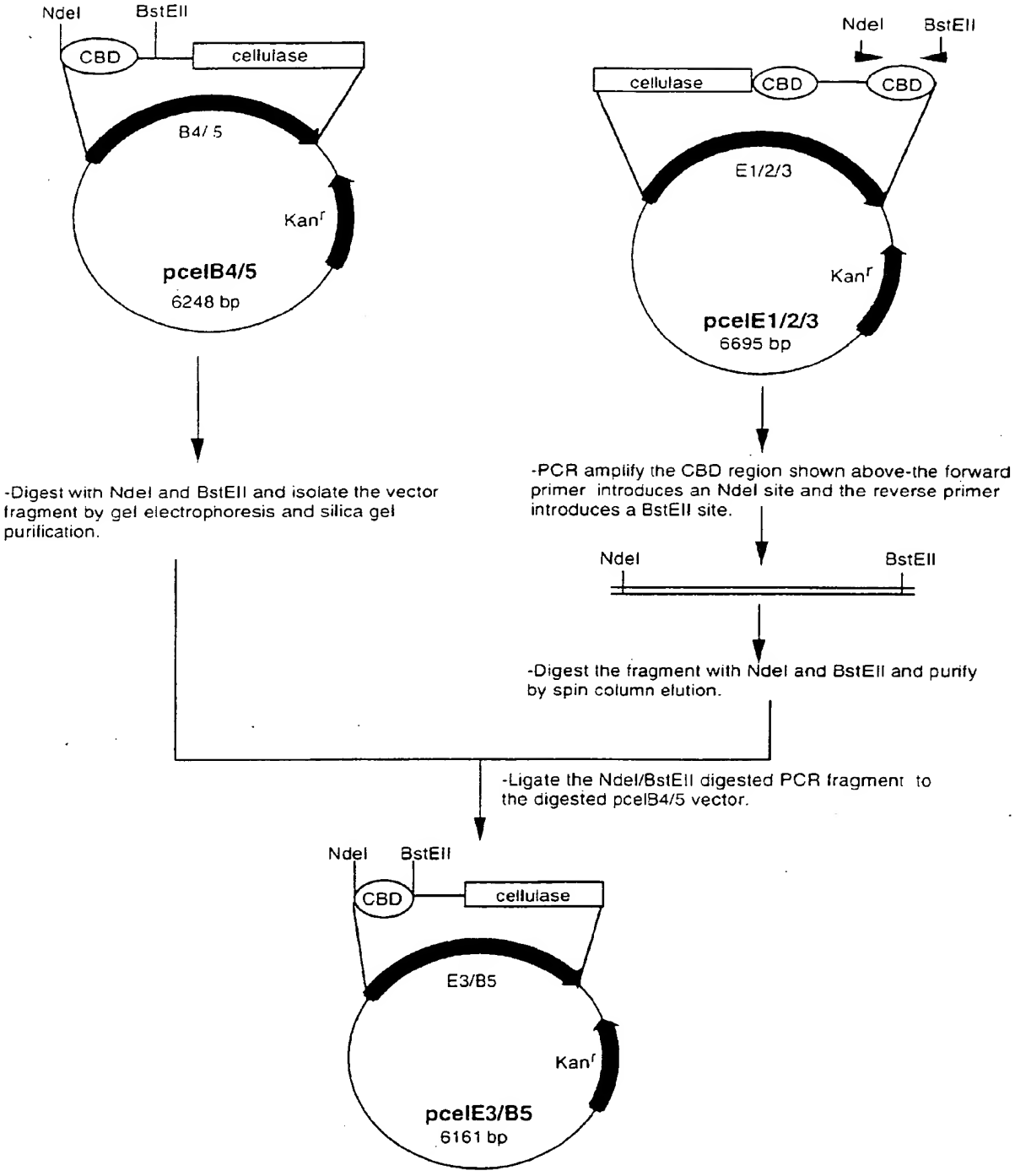


Figure 15.

Sequence Analysis of the Cloned Cellulases

Cellulase Construct	N-terminal Sequence		MALDI-TOF Analysis	
	Expected	Found	Expected	Found
E1	AAYNYGEA	AAYNYGEA		
E1/2			67,425	67,425 (a) 67,245 (b)
B4/5	MKVWYANG	MKVWYANG (c) (X)PTPTPTP(T)I (d)		
B5	ATPSTPTPS	ATPSTPTPS	48.991	48,691(e)

(a) N-terminal amino acids were changed from GT→ AA inorder to facilitate cloning of the protein and based on the found N-terminal sequence of the protein.
(b) N-terminal clipping of the two alanines would result in a 179 dalton decrease in the molecular weight of the protein.
(c) Sequences gave approximately equal picomolar quantities of signal.
(d) Internal cleavage site matching a site in the P-T linker region. (X) indicates that there was no amino acid was detected during the first cycle.
(e) C-terminal clipping of the the final two amino acids, lysine and asparagine, would give the correct molecular weight of 46,691.

Table I.

Oligonucletotide primers designed and synthesized for PCR amplification, genomic walking, and sequencing of cellulase genes from Tok7B.1.

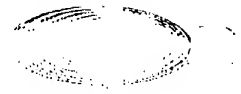
Primer Name	Seq #	Nucleotide Sequence	Length
avicelr	21	5'- TGTATCCCATGCCGTCTT -3'	18
TokcelA	22	5'- CAAAAAGCAATTATGTTTTATGAATT -3'	26
celagwr	23	5'- TGGTGCTGGCAATGTTGAGTTGGC -3'	24
celagwr2	24	5'- TCGGTAGTGCCACTTTCAAATCCA -3'	24
celasf	25	5'- CAAAGCAGACGAATCTGT -3'	18
celasr	45	5'- GCGTGGTATGCAATATAC -3'	18
celbcbd1f	26	5'- AGCTGAGCAGCGGAGTGA -3'	18
celbcbd1r	27	5'- TCCACTCACTCCGCTGCT -3'	18
celbd5r	28	5'- GTTCTGATACTGTCCAAG -3'	18
celekpn	29	5'- ACAGGCGGCGTACAACAT -3'	18
celggwf	30	5'- TTGAGGGATATGGTGACC -3'	18
celhgwf	31	5'- GAGAAACATATCCTGCAA -3'	18
celhgwr	32	5'- CCCATTTTATACCCAGGC -3'	18
celhgwr2	33	5'- TCTTGAGCAGCCATTGGA -3'	18
n17a	34	5'- GATGGCCAGTTCACGTTTATATGG -3'	24
tokcelegwf	35	5'- AGCACTGGTTGGTGGTCCTGGTAG -3'	24
tokcelgwfii	36	5'- GATTGACGGGTTACAATTGGGAGAAC -3'	26
tokcelr	37	5'- AGWGCACCNACAAATCCGGCATTGTARTC -3'	29
tokgw1	38	5'- CTCCAGAATGTCATTTGTAAGATACAT -3'	27

Table II.

Oligonucletotide primers designed and synthesized for PCR amplification and directional cloning of cellulase genes from Tok7B.1. The sequence number for each primer is shown in column two.

Primer Name	Seq #	Nucleotide Sequence/ Engineered restriction sites (Reverse text)	Length	Target Gene	Restriction site	Orientation
celar	3	5'- CCTTTATGAATTCATTTACTGACTGCTA-3'	28	celA	*Eco*RI	Reverse
celcr	4	5'- CTTCCCTCGAGAATTCACACACCCACTTTTG-3'	31	celC	*Xho*I, *Eco*RI	Reverse
celdr	5	5'- TACCCCTCGAGAATTCCTATTTACTCATTA-3'	30	celG	*Xho*I, *Eco*RI	Reverse
celed6f	6	5'- CTACACCCATGGTAACCCCCGATGTTAA-3'	28	celE	*Nco*I	Forward
celff	7	5'- AAATGCTCGAGTAAAAGTGAACAAGCA-3'	27	celF	*Xho*I	Forward
celgf	8	5'- ATGTGTCCATGGCATTAATTATTTTTGTTG-3'	30	celC	*Nco*I	Forward
tok7bcelef	9	5'- ATGCAAGGCATGCAAGCAATTAAGAGGGTTG-3'	31	celE	*Sph*I	Forward
tok7bceleri	10	5'- TCAACAAAGATCTAATCATTTGTGGGTGTTTC-3'	32	celE	*Bgl*II	Reverse
tokcbdxsr	11	5'- GTGCAGCTCGAGCTCCTCCCGGCTCCTGCCCCCA-3'	34	celA-celH	*Xho*I, *Sac*I	Reverse
tokcbdf	12	5'-GAGGAACGGTCATATGAAGGTATGGTATGCGAATGGGAA-3'	39	celA-celH	*Nde*I	Forward
tokcbdfsph	13	5'-GAGGAGGAGCATGCAGATCAAGGTATGGTATGCGAATG -3'	38	celA-celH	*Sph*I	Forward
tokcelb3	14	5'- TTTAGCATGCTGAGGAAATACAAAG -3'	25	celB, celF	*Sph*I	Forward
tokcelbf	15	5'- AGTTAGTGGCATGCAAAAGAGAGTTTTAAGG -3'	31	celB	*Sph*I	Forward
tokcelbr	16	5'- GAAGTATGGATCCATTTATTAATTCTTTGGG -3'	31	celB	*Bam*HI	Reverse
tokceldf	17	5'- TACAATTTTAGCCATGGTAACATACTTTTTAG -3'	35	celD	*Nco*I	Forward
tokceler3	18	5'- GCAGCAGTGTCGACATTTTTATTCTTTAATCTAC -3'	34	celE	*Sal*I	Reverse
tokcelerii	19	5'- GTGGATGAGATCTAACCCGGCTCTAAACCCCA -3'	32	celE	*Bgl*II	Reverse
tokcelhf	20	5'- TTGAACTTCCCCATGGCAGAATTTTTACAAATTGG -3'	35	celH	*Nco*I	Forward
tokcbdf	39	5' - GGGAATTCCATATGGCGGCGTATAATTACGGTGAG -3'	35	celE	*NdeI*	Forward
tokcelecf	41	5' - CCAGAGTATCACAGACAC -3'	18	celE	*none*	Forward
tokcelebamr	42	5' - CCTGGATCCCTACGCTCCTCCCGGCTC -3'	27	celE	*BamHI*	Reverse
tokcel	40	5' - TATTATTATCATATGCGG -3'	15	celE	NdeI	Reverse

Table III. Gene constructs expressed in E. coli by a T-7 promoter.

Gene(1)	Gene Seq ID #	Protein (2) Designation	Domains Expressed	Amino Acids	Protein Seq ID #	Genetic Domains
cel E	2	CelE1		MAAY39-D481 (3)	44	D2
celE	2	CelE1/2		MAAY39-G635 (3)	44	D2/3
celE	2	CelE1/2/3		MAAY39-G812 (3)	44	D2/3/4/5
celB	1	CelB4/5		MK635-N426 (4)	43	D7/8/9
celB	1	CelB5		A1001-P424	43	D9

(1) Gene from which the clone was originally isolated.
(2) Designations of the expressed proteins.
(3) The MAA amino acids contained in the expressed proteins. The single amino acid designations reflect changes in the amino acid sequence resulting from incorporation of a NdeI restriction site at the start of the sequence.
(4) The M is preceeding the gene is a result of the addition of an AUG start codon for expression in E. coli.

Table IV.

Genes constructed	Protein Construct Purified	Thermal Stability °C[1]	pH rate profile [2]	Stonewash Effect
E1	E1	55	5-9	+
E1/2	E1/2	80	4-11	+
E1/2/3	E1/2/3	ND	4-11	-
B4/5	B4/5	55	4-10	-
-	B5	70	4-10	+

(1) Thermal Stability - the highest temperature at which the protein maintains 100% of it's activity for 45 minutes at pH 7.0.
(2) The protein maintains greater than or equal to 20% of it's maximum activity at 50° C.
ND= not determined

(19) Europäisches Patentamt
European Patent Office
Offic européen des brevets

(11) EP 0 921 188 A3

(12) EUROPEAN PATENT APPLICATION

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(54) Genes encoding truncated cellulases and their use

(57) Alkalophilic and thermophilic cellulases having high stability to elevated temperatures and pH have been isolated from an organism of unknown species, which most closely resembles those in the *Caldicellulosiruptor* genus and which has been called by us, Tok7B.1. These cellulases have been cloned and expressed in a recombinant system, so that they can be produced in quantity. These are particularly useful in treating cellulosic materials including cotton-containing fabrics, as detergent additives, and in aqueous compositions. We also provide genomic DNA which can be used in recombinant expression vectors and expression systems to produce enhanced alkali and/or temperature stability properties in cellulases other than those specifically described.

Figure 2.

Blast sequence homology search with the identified N-terminal peptides shows the proteins have homology with Families 9 & 10 from Glycosyl hydrolases. Areas of homology between sequenced N-termini are shown in black backgrounds with white lettering.

Peptide No.	Amino-terminal amino acid sequence	Glycosyl Hydrolase Family based on amino acid homology comparisons
B1	A[redacted]KAIMFYEFXM	
B3	A[redacted]	Glycosyl hydrolase
B5	G[redacted]	Family 9
B6	G[redacted]	
B2	[redacted]ESKYND	Glycosyl hydrolase
B4	[redacted]	Family 10

EP 0 921 188 A3

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EUROPEAN SEARCH REPORT

Application Number
EP 98 81 0919

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
A	DATABASE EM_PRO 'Online! EMBL; 16 February 1989 (1989-02-16) SAUL D.J.: "Caldocellum saccharolyticum celB gene for cellobiohydrolase/endocellulase" retrieved from EBI, accession no. CSCELB Database accession no. X13602 XP002205070 * the whole document *	1,7	C12N9/12 C12N15/55 C11D3/386 D06M16/00 D06P5/04 D06P5/13 D06P5/15 C12N9/42 D06P5/02
A	-& DATABASE SWALL 'Online! 1 July 1989 (1989-07-01) SAUL ET AL.: "Endoglucanase/exoglucanase B precursor ...." retrieved from EBI, accession no. GUNB_CALSA Database accession no. P10474 XP002205071 * the whole document * & NUCLEIC ACIDS RES., vol. 17, 1989, page 439	1,7	
A	DATABASE EM_PRO 'Online! EMBL; 30 November 1994 (1994-11-30) MORRIS ET AL.: "Caldocellum saccharolyticum endoglucanase/mannase celC) pseudogene, complet cds." retrieved from EBI, accession no. CS16308 Database accession no. U16308 XP002205072 * the whole document * & APPL ENVIRON MICROBIOL, vol. 61, no. 6, 1995, pages 2262-2269,	1,7	

-/--

TECHNICAL FIELDS SEARCHED (Int.Cl.6)
C12N
C11D
D06M
D06P

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
BERLIN	8 July 2002	Ceder, O

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X : particularly relevant if taken alone
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& : member of the same patent family, corresponding document

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EUROPEAN SEARCH REPORT

Application Number
EP 98 81 0919

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
A	DATABASE SWALL 'Online! 1 August 1991 (1991-08-01) GIBBS ET AL.: "Beta-mannanase/endoglucanase A precursor ...." retrieved from EBI, accession no. MANB_CALSA Database accession no. P22533 XP002205073 * the whole document *	1,7	
A	-& APPL. ENVIRON. MICROBIOL., vol. 58, 1992, pages 3864-3867, XP000618236 ---		
A	DATABASE SWALL 'Online! 1 May 1997 (1997-05-01) ZVERLOV V.: "Endoglucanase A (EC 3.2.1.4) ...." retrieved from EBI Database accession no. P96311 XP002205074 * the whole document * ---	1,7	TECHNICAL FIELDS SEARCHED (Int.Cl.6)
A	DATABASE SWALL 'Online! 1 May 1997 (1997-05-01) ZVERLOV V.: "Endo-1,4-beta-glucanase (EC 3.2.1.4) (Fragment)" retrieved from EBI Database accession no. P96310 XP002205075 * the whole document * ---	1,7	
A	WO 96 23928 A (GENENCOR INT) 8 August 1996 (1996-08-08) * abstract; claims * * page 5, line 38 - page 7, line 6 * * page 19, line 8 - line 21 * --- -/--	1-13	

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
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Application Number
EP 98 81 0919

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
A	WO 95 16782 A (GENENCOR INT) 22 June 1995 (1995-06-22) * abstract; claims * * page 4, line 15 - page 5, line 11 *	1-10	
A	WO 94 29426 A (GENENCOR INT) 22 December 1994 (1994-12-22) * abstract; claims *	11,12	
A	TE'O V S J ET AL: "CELA, ANOTHER GENE CODING FOR MULTIDOMAIN CELLULASE FROM THE EXTREME THERMOPHILE CALDOCELLUM SACCHAROLYTICUM" APPLIED MICROBIOLOGY AND BIOTECHNOLOGY, SPRINGER VERLAG, BERLIN, DE, vol. 43, no. 2, May 1995 (1995-05), pages 291-296, XP001057912 ISSN: 0175-7598		
A	TYNDALL R M: "IMPROVING THE SOFTNESS AND SURFACE APPEARANCE OF COTTON FABRICS AND GARMENTS BY TREATMENT WITH CELLULASE ENZYMES" TEXTILE CHEMIST AND COLORIST, AM. ASSOC. OF TEXTILE CHEMISTS AND COLORISTS. NORT CAROLINA, US, vol. 24, no. 6, 1 June 1992 (1992-06-01), pages 23-26, XP000278729 ISSN: 0040-490X		TECHNICAL FIELDS SEARCHED (Int.Cl.6)

-/--

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
BERLIN	8 July 2002	Ceder, O

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& member of the same patent family, corresponding document

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DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
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The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
BERLIN	8 July 2002	Ceder, O

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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